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UNIVERSITY OF ALBERTA

A STUDY OF MISMATCH REPAIR DURING RECOMBINATION IN *Saccharomyces cerevisiae*

BY

P. MANIVASAKAM

©

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF GENETICS

EDMONTON, ALBERTA

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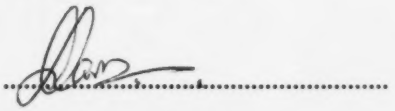

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The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies for acceptance, a thesis entitled A study of mismatch repair during recombination in *Saccharomyces cerevisiae* submitted by P.Manivasakam in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

A heteroduplex intermediate is formed during recombination. If mismatches in the heteroduplex are corrected, the result is either conversion or restoration. Postmeiotic segregation (PMS) results if the mismatches are not corrected. This project was undertaken to study mismatch repair during recombination in yeast. Alleles giving a high frequency of PMS (PMS alleles) were used to examine the interaction of PMS alleles with normal alleles (those giving a low frequency of PMS) in the mismatch repair process. A variety of defined alleles (both PMS and normal alleles) were constructed at the *HIS1* locus by *in vitro* mutagenesis and replacement of the wild-type allele. Crosses were made with these mutations at defined distances between alleles. Intragenic recombination rates and frequencies were calculated in mitotic and meiotic cells. The crosses between a PMS and a normal allele showed hyper-recombination (HR) if the distance between the two alleles was short (between 8 and 20 bp). A cross with two normal alleles at the same distance did not show HR. In this study, three types of PMS alleles were tested: (i) G to C transversions, (ii) a deletion and (iii) a palindromic insertion. All showed HR in two-point crosses as long as the distance between the PMS allele and normal allele was short. Analysis of selected and unselected tetrads and selected prototrophs showed that HR is associated with postmeiotic segregation.

Based on these results a molecular model is proposed to account for the HR phenomenon in yeast. It is suggested that postmeiotic segregation results either from blockage of mismatch repair after the recognition step, or by interference of some other DNA binding protein(s) which block access of the mismatch repair machinery to carry out repair. The model proposes that, normally, repair is bidirectional at the normal alleles. HR is due to postmeiotic segregation of PMS alleles which in turn makes repair at the normal allele unidirectional. The unidirectionality of mismatch repair originating from the normal allele in HR crosses was verified with three point crosses

involving two PMS alleles and a normal allele. My results show that a high PMS allele blocks co-repair of alleles flanking it.

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ABBREVIATIONS

bp: base pair(s),

BSA: bovine serum albumin,

dATP: 2'-deoxyadenosine-5'-triphosphate,

dCTP: 2'-deoxycytidine 5'-triphosphate,

dGTP: 2'-deoxyguanosine 5'-triphosphate,

DTT: dithiothreitol,

dTTP: 2'-deoxythymidine 5'-triphosphate,

EDTA: ethylene diamine tetra acetic acid,

kb: kilo base(s),

NMS: non-Mendelian segregation,

PCR: polymerase chain reaction,

PEG: polyethylene glycol,

PMS: postmeiotic segregation,

SDS: sodium dodecyl sulphate,

TE (1x), pH: 8.0: 10mM Tris. HCl (pH: 8.0), 1 mM EDTA.

Introduction

Recombination is the production of new combinations of alleles by transfer of genetic information from one DNA molecule to another. These new genetic combinations have many consequences, from evolution to the life of an individual organism. In evolutionary terms, mutations are the primary source of variation upon which natural selection acts and selects the favorable mutations. Recombination accelerates the evolutionary process by bringing the favorable mutations of different organisms together in one organism. In a few cases, for example, insertion of transposable elements and translocations of chromosomes, mutations are caused by the recombination process itself.

Recombination plays an essential role in the biological functions of individual organisms. Recombination is commonly concerned in ensuring the accuracy of chromosome segregation during meiosis because it provides the mechanism for formation of chiasmata which hold bivalents together on the metaphase plate of the first meiotic division. Mutants that are defective in recombination lack chiasmata and produce meiotic products most of which are aneuploids. Even more important to the every day life of an organism is the fact that recombination provides one of the major mechanisms of DNA repair. Recombination-deficient mutants such as *recA* in *Escherichia coli* and *rad* mutants in yeast are extremely sensitive to DNA-damaging chemicals and ionizing radiation. Recombination is also involved during differentiation in the programmed rearrangement of the genome. It plays a major role in phase variation in *Salmonella*, antigenic variation in trypanosomes and borelia, generation of antibody diversity in vertebrates, and in mating type switching in yeast. Recombination also plays an important role in the unprogrammed rearrangement of the genome such as gene amplification and diminution, and chromosomal translocations.

The traditional meaning of the term recombination has been the formation of new combinations of alleles in the progeny that differ from the parental combinations. Although recombination processes take place in both mitotic and meiotic eukaryotic cells the frequency of recombination is many fold higher in meiosis than in mitosis. The high rates of recombination in meiosis have resulted in an historical preference for meiosis in the study of recombination.

Much of our understanding of the mechanism of recombination is based on the studies of fungi such as *Ascobolus*, *Neurospora*, *Sordaria* and Yeast (*Saccharomyces cerevisiae*) in which four meiotic products can be analyzed relatively easily. Though the studies of these fungi has yielded a wealth of information at the level of the gene, many molecular details have come from studies in prokaryotic systems like *E. coli* and its phages as the molecular biology systems of these organisms are well advanced. But not all information about recombination mechanisms obtained in prokaryotes can be extrapolated directly to eukaryotes due to differences in life cycle between these two classes of organisms which are separated widely in evolution. As yeast has the advantage of having both good classical genetics and well developed molecular systems, this simple eukaryote has become a favorite organism for the study of recombination in recent years.

In eukaryotes, the genes located on different chromosomes recombine by independent assortment; genes located on the same chromosome recombine predominantly by a reciprocal process (breakage and joining) called crossing-over. Alleles of the same gene are often observed to recombine by a non-reciprocal process called conversion. Because reciprocal and non-reciprocal recombination processes are correlated they are considered to be different outcomes of the same initial process. The results of numerous studies in yeast and other organisms show that conversion (the non-reciprocal process) occurs by two distinct mechanisms: (i) the formation and repair of heteroduplex DNA and (ii) double-strand gap filling. It was postulated that a

heteroduplex intermediate is formed during recombination (Holliday, 1962). The presence of the heteroduplex proposed by Holliday was confirmed later in many organisms. The heteroduplex, once formed, is subject to mismatch repair. If the heteroduplex survives through meiosis it is seen as a postmeiotic segregation (PMS) event and, if repaired, the outcome is either conversion or restoration of an allele. The PMS event itself is taken as evidence for the presence of the heteroduplex intermediate in recombination. Recombination is a stepwise process involving initiation, formation of intermediates, processing and resolution of the intermediates. As most intragenic recombination occurs by conversion, mismatch repair plays an important role in recombination and hence the outcome of recombination may vary with mismatch repair. The major goal of this study is to extend our understanding of the mismatch repair process in recombination. Alleles giving a high frequency of PMS (PMS alleles) were used to examine the interaction of PMS alleles in the mismatch repair process.

Intragenic recombination studies of the *CYC1* locus of yeast (Moore et al., 1988) show 50 to 100 fold increased level of recombination (hyper-recombination= HR) in two-point crosses involving a G to C transversion mutation allele as one of the alleles in the cross with any other point mutation as the other allele. This HR effect was only seen when the distance between two mutational sites was between 4 and 20 base-pairs. In yeast, other alleles with a G to C transversion show postmeiotic segregation. Based on these observations we postulate that the G to C mutations at *CYC1* are PMS alleles and that the HR phenomenon is due to proximity of the PMS and normal alleles in these crosses.

In the first part of this study, the above hypothesis is tested by addressing the following questions:

1. Do PMS alleles show hyper-recombination with normal alleles ?
2. Is the hyper-recombination phenomenon distance dependent ? and

3. Is hyper-recombination associated with PMS ?

The approach to addressing the above questions involved the construction of PMS and non-PMS mutant alleles at specific sites in the *HIS1* gene. Consequently, crosses could be made using mutants with defined mutational types that are separated by various distances. The outcome of recombination events could then be studied to establish a causal relationship between the mutational types, the distance between them and the various types of recombination events. The results show that HR is observed in crosses involving any PMS allele and a normal allele within a short distance. This HR is associated with PMS. A molecular model is proposed for the HR phenomenon.

In the second part of this study, I tested the molecular model with a variety of three-point crosses involving two PMS alleles and a normal allele. The results and implications of these experiments are presented here.

Review of literature

1. Reciprocal and non-reciprocal exchange

In eukaryotes, recombination occurs in two ways: reciprocal (crossover) and non-reciprocal (conversion). Reciprocal recombination is seen as an exchange of markers between homologous chromosomes. Morgan introduced the term crossing-over for the process of interchange by which new combinations of linked factors arose (Morgan and Cattell, 1912). Janssens (1909) observed nodes or chiasmata in meiosis and he postulated that the nodes represent the point of exchange. This correlation was established by Creighton and McClintock (1931). Janssens (1909) suggested that the exchange takes place only between one of the two chromatids from each parental chromosome. This would explain the cytological observation of involvement of only two chromatids. The breakage and joining process of crossing-over yields the reciprocal exchange of information.

In crosses between a wild-type and a mutant, a single marker usually segregates 2:2 among the four meiotic products. In the octad, one more mitosis produces 8 spores (4:4). This is called Mendelian segregation. Zickler (1934) studied *Bombaria lunata* mutants affecting spore colour. He found that in asci heterozygous for a mutation giving rise to a pale ascospore, the majority of asci display Mendelian segregation, but, occasionally, asci with six dark (wild-type) and two pale spores, or two dark and six pale ones are observed. He used the term conversion for this non-Mendelian segregation. Lindegren (1953) found 3:1 ratios in *Saccharomyces cerevisiae* tetrads. Later studies showed that these conversions are not due to mutations but occur by recombination. In these events the alleles have been recombined non-reciprocally. The convention for reporting ascus genotype is to list wild-type then mutant, thus 2:6 means two wild-type to six mutant spores.

Olive (1956) studied spore colour mutants of *Sordaria fimicola*. Heteroallelic crosses give four kinds of aberrant asci: 6:2, 2:6, 5:3 and 3:5. A fifth kind of aberrant segregation (aberrant 4:4) was discovered by him in a cross between wild type and a grey-coloured spore mutant. The 5:3, 3:5 and 4:4 categories of aberrant segregation show segregation of a marker after meiosis. This is called postmeiotic segregation (PMS). After Olive's discovery of PMS, similar findings were reported in many fungi: *Ascobolus, Neurospora and Saccharomyces cerevisiae*. Kitani et al, (1962) studied the relationship of aberrant non-Mendelian segregation and crossing-over in a spore colour locus that is flanked by linked genes. Their analysis of asci showing aberrant segregation showed a positive correlation between crossingover and aberrant segregation. About 40% of the aberrant segregation events have associated crossovers in *Ascobolus* (Stadler et al., 1970). The crossovers are observed only on the chromatids showing aberrant segregation. The association of crossing-over with aberrant segregation has been noticed in many organisms. In *S. cerevisiae*, (compiled by Fogel et al., 1981) it ranges from 18 to 66%. The buff locus in *Sordaria* (Kitani and Olive, 1969) and *met-7* in *Neurospora crassa* (Murray, 1969) show 20 and 15%, respectively. Postmeiotic segregation and gene conversion have the same frequencies of associated crossovers at *SUP6* (DiCaprio and Hastings, 1976). This association suggests that aberrant segregation (PMS plus conversion) and crossing-over result from a common initial event. Both PMS and conversion are recombination events. Reciprocal recombination can be seen with a minimum of two markers, but conversion can be seen with a single marker. In general most inter-genic and some intra-genic recombination is due to reciprocal exchange, whereas most intragenic recombination is by non-reciprocal events.

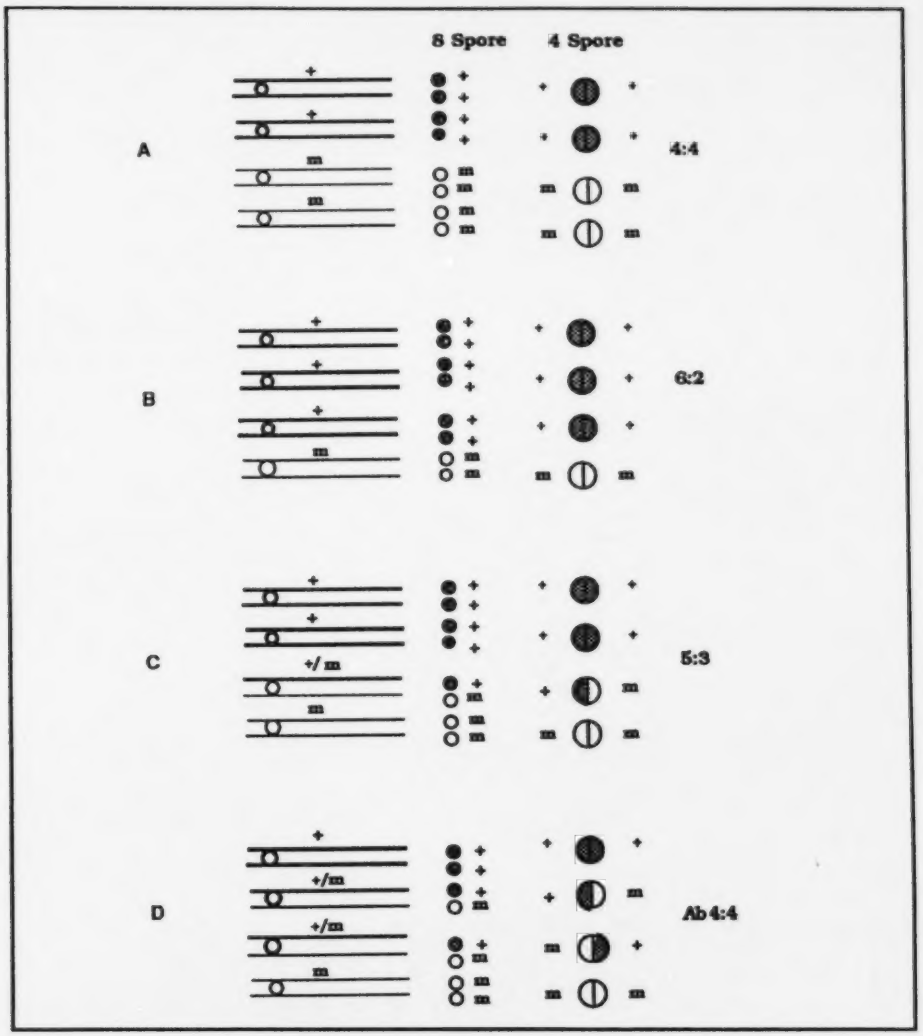

FIG. 1. Segregation patterns in the fungi, producing either 8 or 4 spores.

Each single line indicates a single DNA strand.

A. A heterozygous marker, +, will normally segregate 2+:2m or 4+:4m.

B. Occasionally the marker segregates 3+:1m or 6+:2m-a gene conversion event.

C. A postmeiotic segregation event, 5+:3m, is detected after one round of postmeiotic DNA synthesis and produces a sectored colony in fungi with four spores.

D. If heteroduplex DNA is present on two chromatids and no mismatch correction occurs, an aberrant 4+:4m segregation results. In the four-spored fungi an aberrant 4:4 segregation gives two sectored colonies.

2. Heteroduplex and aberrant ratios

Aberrant ratios cannot be explained by simple breakage and rejoining of DNA molecules. Holliday (1962) proposed that crossing-over occurs by breakage and joining and that aberrant segregation is a different outcome of the same process. He suggested that recombination takes place by annealing of complementary nucleotide chains, derived one from each parent, to produce heteroduplex or hybrid DNA. The repair of base mismatches in the heteroduplex could give rise to various types of aberrant ratios (FIG. 1). As shown in the figure, if there is no heteroduplex, the resulting spores after meiosis show normal Mendelian segregation (4:4 or 2:2). A 5:3 postmeiotic segregation occurs if heteroduplex DNA formed on one chromosome is not repaired. Aberrant 4:4 segregation is seen if heteroduplex is formed and is not repaired on two chromatids. Conversions (6:2) may arise from mismatch repair in heteroduplex DNA. This hypothesis gives a simple explanation for the various types of aberrant segregation observed in fungi. There are alternatives to this proposal that will be discussed later. After the suggestion of a heteroduplex intermediate by Holliday (1962), the presence of heteroduplex has been shown in many systems. PMS is taken as evidence for the presence of a heteroduplex intermediate in recombination (Holliday, 1962). The PMS event shows that two alleles segregate from a spore that contained two types of information in duplex DNA. In this heteroduplex intermediate model, if the mismatch in the heteroduplex survives through meiosis, it is seen as PMS. If there is mismatch correction, this is seen as conversion, or is not seen because it yields restoration. Direct evidence that heteroduplex is a precursor to conversion and PMS is seen in the repair defective mutants (*pms1*) of yeast. In these mutants the frequency of PMS increases and conversion decreases without increasing the frequency of non-Mendelian segregation (Williamson et al., 1985; Reenan and Kolodner, 1992).

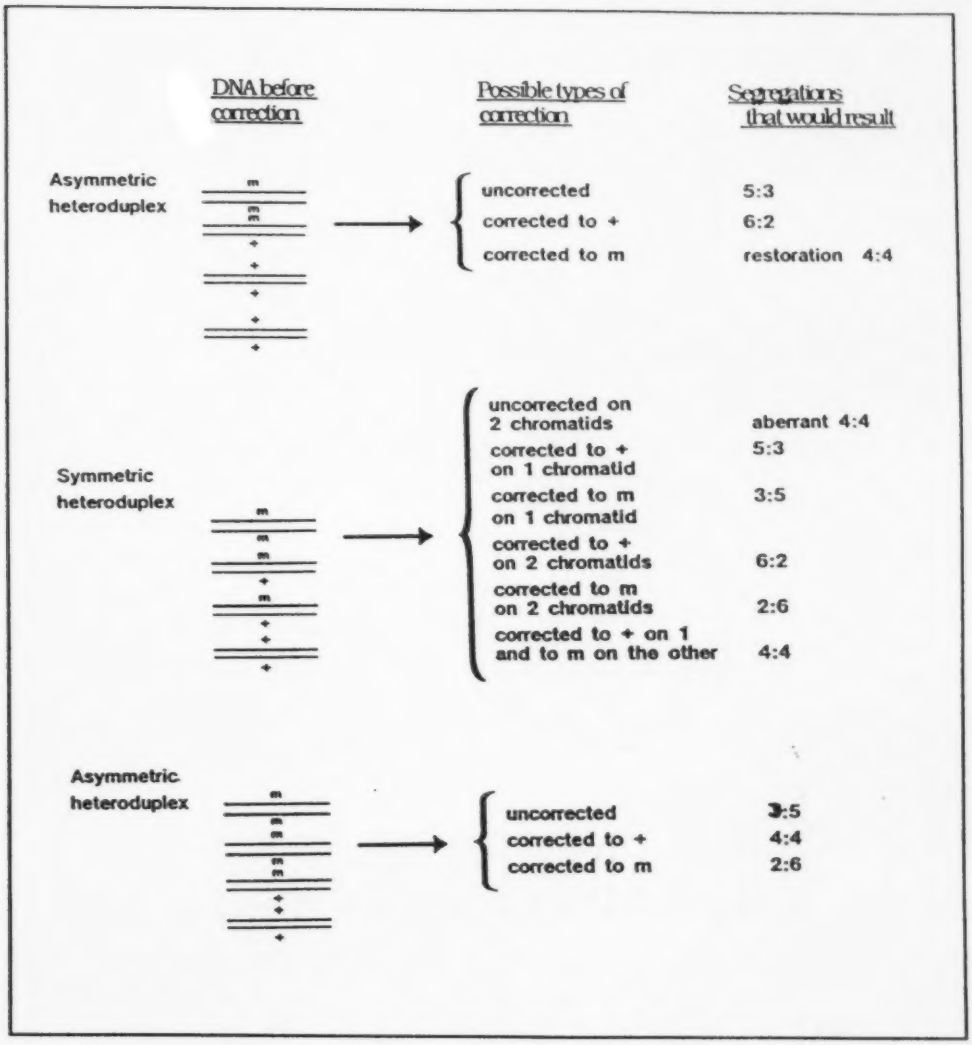

FIG. 2. Origin of various non-Mendelian segregation ratios on the mismatch correction hypothesis of conversion (reviewed by Hastings, 1988). The number of wild type: mutant spores is listed in the ratio.

Conversion spectrum

The pattern of segregation ratios shown by a marker is known as its conversion spectrum. The relationship between segregation pattern and type of allele has been studied by Leblon (1972 a, b). He analyzed the aberrant segregation patterns of mutant alleles affecting spore colour (b2 locus) in *Ascobolus*. Most of the alleles could be grouped into three classes: A, B and C. Some markers show little or no PMS, and give an excess of 6+:2- over 2+:6- (class A), showing conversion to wild type more often than to mutant. The B type alleles have the opposite disparity, showing an excess of 2:6 over 6:2. The third class (C) alleles show some postmeiotic segregation events. All three classes of alleles occur in the b1 and b2 loci. Frame shift mutations are A and B types of alleles. Single base-pair (bp) insertion mutations give an excess of conversion towards mutant, whereas one bp deletions give an excess towards wild-type. Base substitutions come under class C and show high PMS. Most large deletions show parity: conversion to wild type or to mutant is approximately equal. However, some deletions show strong disparity and are preferentially converted to wild-type (parity and disparity are discussed later). The relative proportion of different ratios of a marker can be explained by heteroduplex formation and repair. The frequency of tetrads showing different ratios depends on many factors: (i) whether the heteroduplex is formed on one or two chromatids; (ii) whether the mismatch in the heteroduplex is corrected or not, and (iii) whether repair is towards wild type or towards mutant information. The relationship between ratios and mismatch formation and repair is given FIG. 2.

The segregation pattern in yeast is different from *Ascobolus*. Almost all mutations, including base substitutions, are corrected efficiently and show low frequencies of PMS (Fogel et al., 1981) in yeast. All mutations show approximate parity in conversion frequency of 3:1 and 1:3 (6:2=2:6). There are only three classes of alleles that show PMS in yeast (discussed later). In *Schizosaccharomyces pombe* also, most mutations show parity (Thuriaux et al., 1980). There are exceptions. The M26 mutation

in the *ade6* locus of *S. pombe* shows extreme disparity, 3:1 segregation being observed 12 times more often than 1:3. The above evidence shows that conversion spectra are allele-specific. As discussed later, they are also position-specific and locus-specific.

3. Heteroduplex distribution

A. Polarity

The frequency of aberrant ratio tetrads exhibited by a marker is called the frequency of non-Mendelian segregation (NMS frequency = Frequency of conversion+postmeiotic segregation). In *Ascobolus*, Paquette and Rossignol (1978) analyzed 15 class C mutants (showing mainly PMS) that spanned the *b2* locus. The frequency of all aberrant segregation events was highest for alleles on one side of the gene (left side) and decreased towards the other side. This type of gradient is seen in A and B type alleles too (Paquette and Rossignol, 1978). The total frequency of aberrant segregations also decreases across the locus in many genes of *S. cerevisiae*. The range, referred to as polarity, is from 8.3% to 2.5% in *ARG4* (Fogel et al., 1981) and 2.9% to 1% in *HIS1* (Savage and Hastings, 1981).

Polarity is also seen in intragenic recombination between two mutations. Prototrophs from the unselected tetrads show that in a series of two-point crosses, the allele toward one end of the locus is the one to be converted most in each cross (Lissouba et al., 1962). One interpretation of polarity is that heteroduplex is initiated near one end of the gene and ends at different positions within the gene. The alleles close to the putative initiation site are included in the heteroduplex more often than the ones distant from the site. Thus, a gradient in NMS frequency from one end to the other could result. In the *b2* locus, the polarity in relation to transcription is not known. In the *ARG4* locus of yeast, the polarity is from 5' to 3' (Nicolas et al., 1989), but in *HIS1* (Savage et al., 1989) and *HIS3* (Malone et al., 1992) it is opposite (3' to 5') . In *ARG4* the putative initiation site of recombination is located at the 5' end of the gene. Deletion

analysis identified a 142 bp sequence at the promoter site (Nicolas et al., 1989). Homozygous deletion of the site lowers the frequency of conversion and abolishes the polarity gradient on both sides of the putative initiator.

For most genes the NMS frequency decreases from one end to the other. However in the *lysF* gene of *Aspergillus nidulans* (Pees 1967), NMS is high on both sides of the gene and produces a U-shaped pattern. This could be explained by the existence of initiation sites on both ends of the gene. Another explanation for polarity was offered by Detloff et al. (1992). They proposed that gradients in NMS frequency reflect the direction of mismatch repair rather than the frequency of heteroduplex formation. Using PMS alleles, they measured the frequency of heteroduplex formed at *HIS4*. They showed that there is not much variation in the frequency of heteroduplex from 5' to 3'. NMS frequency of normal alleles shows a gradient of conversion from 5' to 3'. They interpret this result to mean that at the high conversion end, most of the repairs are conversion types and at the low conversion end they are restoration types.

B. Symmetry and asymmetry

Crossing-over in meiosis involves two of four chromatids. Intragenic recombination associated with crossing-over of flanking markers often reveals that a chromatid involved in a crossover shows PMS. But this PMS is not always found on both chromatids. Symmetry is revealed by the presence of aberrant 4:4 segregation and asymmetry by 5:3 and 3:5 segregation. Paquette and Rossignol (1978) showed that, in the b2 locus, the frequency of aberrant 4:4 segregations increased for C- class alleles towards the low conversion side of the gene. This indicates that the low conversion end of the gene contains more symmetrical heteroduplex DNA than the high conversion end does. In yeast the frequency of tetrads showing PMS is low: 0.15% of unselected tetrads (Fogel et al., 1981). The most common type of PMS is 5:3 or 3:5. Aberrant 4:4 segregations are very rare in yeast. So it was assumed that heteroduplex formation in

yeast usually involves only a single chromatid (asymmetrical) rather than the symmetrical involvement of two chromatids (Fogel et al., 1981).

4. Heteroduplex correction

Once heteroduplex DNA is formed, it is subject to mismatch repair as shown in FIG. 2. Correction can produce either conversion or restoration. Non-repair yields postmeiotic segregation. In eight-spored asci this is seen as 5:3 or 3:5 ratios or aberrant 4:4. In four spored asci (tetrads), as in yeast, this is seen as a sectored colony displaying two phenotypes.

A. Postmeiotic segregation (PMS)

Postmeiotic segregation is detected phenotypically as the sectoring of a single heterozygous marker in a haploid ascosporal colony. This represents marker segregation at the first postmeiotic mitosis. PMS has been reported in many organisms such as *Ascobolus*, *S. cerevisiae* and *S. pombe*. PMS in yeast was observed by Esposito (1971). In yeast PMS events are much rarer than gene conversion events, occurring at frequencies of between 0.5 and 5% of unselected tetrads (Fogel et al., 1981). Only three types of alleles show PMS in yeast: (i) some deletions (*his1-49* and a*de8-18*), (ii) all G to C transversion mutations, and (iii) all palindromic insertions.

In the *ADE8* gene the mutant allele displaying the highest PMS frequency in an *ade8/ADE8* heterozygote (*ade8-18*) was determined to be a 38-bp deletion (White et al., 1988). White et al. (1988) further tested whether small deletions in yeast will give PMS. They made a series of deletion mutations in the *ADE8* gene in *S. cerevisiae*. PMS was observed for a subset of the deletion heterozygotes, including deletions of 38 to 93 bp. They found that there was no clear relationship between deletion length and PMS frequency. They suggested that a common sequence can be identified in the flanking regions of the heteroduplex. This sequence is related to a repeated sequence associated

5'-A G G A C G G C A G G C A-3'
Mouse I J repeat
5'-A G G T G G G C A G G A X-3'
Human mini-satellite
5'-G C P X G G G C Y G G X T-3'
Consensus sequence present at the flanking regions of yeast deletion mutants showing PMS
5'-G C T G G T G G-3'
E.coli Chi sequence
5'-G A T G G A T G A C G T-3'
ade6-M26
5'-G A T G G A G G A C G T-3'
ADE6 Wild type
3'-G A C G G G C G A C G T-5'
his1-49+ sequence
P=Purine
Y=Pyrimidine
X=Any base

FIG. 3. Sequences associated with recombination hotspots and PMS alleles.

with recombination hotspots in the human and mouse genomes (FIG. 3). They propose that these deletion heterozygosities escape heteroduplex DNA repair because of binding of a protein involved in recombination which blocks mismatch repair. Another deletion that shows a high level of PMS is in the *HIS1* locus. The allele *his1-49* is a 37 bp deletion (Savage et al., 1989). This allele does not have the consensus sequence found in *ade8* deletion mutations. However, it does have a sequence similar to that found in the *ade6-M26* mutation of *S. pombe*. A single base-pair change from G to T in the M26 mutation creates a recombination hotspot. This M26 mutation has increased frequencies of reciprocal recombination and conversion in meiosis only (Ponticelli et al., 1988). A similar sequence is found in the deleted part of the *his1-49* sequence but with opposite polarity (FIG. 3). In heteroduplex DNA, this sequence will be present in the single-strand loop. If this is a protein binding site, and if PMS is the result of protein binding, then the protein might recognize the sequence even in a single-strand.

White et al. (1985) found that the *arg4-16* mutation registers a high level of PMS (48% of aberrant segregation) when heterozygous with wild type. In *HIS4*, Detloff and Petes (1992) recorded 81% PMS using the *his 4-200* allele. Both of them are G to C transversion mutations.

Inserted palindromic sequences in *HIS4* and *LEU2* genes exhibit PMS (Nag et al., 1989). Nag and Petes (1991) showed that at least a 7-bp repeat is necessary to get PMS and imperfect palindromes are repaired efficiently. In the same study 5-and 6-bp repeats gave 10% and 3% PMS, respectively. This is interpreted to mean that palindromic sequences in heteroduplex DNA inhibit mismatch repair and cause PMS.

B. Conversion

Gene conversion is the nonreciprocal transfer of information to both strands of a homologous DNA molecule. Conversion events are detected as 3:1 and 1:3 ratios in tetrads or 6:2 and 2:6 ratio in octads. There is evidence to show that conversion can

occur by two mechanisms: (i) the repair of heteroduplex, and (ii) the repair of double strand gaps. As discussed earlier, at least some conversion reflects mismatch repair in heteroduplex as is seen from *pms* mutants (Williamson et al., 1985) in which the frequency of PMS events is increased at many heterozygous loci without increasing the frequency of non-Mendelian segregation. This is interpreted to indicate that during recombination a heteroduplex intermediate is formed and repair of this heteroduplex gives conversions. This implies that most meiotic gene conversions arise by heteroduplex correction. Orr-weaver et al. (1981) showed, in an experiment involving the integration of gapped plasmids, that the gap was filled-in during integration. This experiment indicates that conversion can also occur without mismatch repair of an heteroduplex intermediate. Though conversion can occur either by heteroduplex correction or by gap filling, most meiotic gene conversions arise by mismatch repair (heteroduplex correction) as discussed before. The conversion frequency depends on two factors: (i) the amount of heteroduplex DNA formed in the gene, and (ii) the way in which the heteroduplex is repaired.

C. Frequency of heteroduplex formation

The frequency of heteroduplex formed for an allele depends on the gene in which it is located. Some genes show more recombination than others. For example at the *HIS4* locus in yeast, the non-Mendelian segregation frequency (which is the measure of recombination frequency of the allele) is up to 40% whereas at *HIS1* the maximum level is only 3% (Savage and Hastings 1981). Thus the genes in yeast can be considered hot (high) or not hot (normal) for recombination.

Although genetic distances are often assumed to be proportional to physical distances, in numerous organisms certain regions or sites called hot spots exhibit an elevated frequency of recombination (reviewed by Whitehouse, 1982). In yeast, for example, the region between *CDC24* and *PYK1* on chromosome I is about five-fold

higher for exchange than an average interval (Coleman et al., 1986), and the *LEU2-HIS4* region of chromosome III is about three-fold higher (Newlon et al., 1986). Some hotspots are hot in mitosis and others are hot in meiosis. The *HOT1* rDNA locus is hot in mitosis only (Stewart and Roeder, 1989) whereas *ade6-M26* is specific to meiosis (Ponticelli et al., 1988). In some cases, transcription also increases recombination. In *HOT1*, transcription is necessary for the elevated level of recombination. Grimm et al. (1991) showed that a strong promoter can also increase the recombination frequency in meiosis. The replacement of the *ADE6* promoter with that from *ADH1* increased meiotic recombination at this locus 20-25 fold in *S. pombe*. The region between *MAT* and *CRY1* on chromosome III is a strong cold spot for exchange (Larkin and Woolford, 1984), as is the region between the two silent mating cassettes in *S. pombe* (Egel, 1984). One of the factors influencing recombination levels could be the presence or absence of initiation sites of recombination at or near the gene. If a gene is located close to an initiation site (*HIS4* and *ARG4*), it may show elevated recombination.

Another factor is the location of the allele within the gene. As seen in polarity gradients, the apparent frequency with which an allele is included within heteroduplex DNA depends on where the allele is located. An allele that is close to the high conversion end is likely to be included in heteroduplex more often than one located at the low conversion end.

As discussed earlier, Detloff et al. (1992) proposed that a polarity gradient reflects the direction of mismatch repair rather than the frequency of heteroduplex.

D. Parity in conversion and restoration

A second factor that influences the frequency of conversion is how the heteroduplex DNA is repaired. During recombination, the same basic event has a different outcome in a tetrad depending on whether the mismatch is converted or restored. In the formation of a mismatch, one strand of the duplex probably invades

the other duplex. If the invading strand is repaired, the original information in the duplex is restored. On the other hand if the invaded strand is repaired, the new information replaces the original one. This latter process is called conversion. If there is no preference in excision of the invading or invaded strand, there will be parity in restoration and conversion. If one genotype or one strand is more likely to be excised than the other this will show disparity (2:6≠6:2). Restorations cannot be detected without special markers or techniques and hence they are counted as non-recombinants. As with the variation of aberrant ratios, parity and disparity are allele-specific.

In yeast almost all alleles show parity in conversion to mutant and to wild type (Fogel et al., 1981). This could be due to an equal probability of correction of heteroduplex in two directions. But this assumption was questioned by Savage (1979) based on intragenic reciprocal recombination in the *his1* locus of *S. cerevisiae*. She proposed that parity in conversion to mutant and to wild type is achieved by an equal and opposite disparity in the excision of donor or recipient information, giving a strong and allele-specific excess of conversion over restoration. In *S. pombe*, the *ade6-M26* recombination hotspot mutation presumably acts as an initiator of recombination. Tetrad analysis involving M26 yields a twelve-fold increase of conversion to wild-type (3+:1m) relative to conversion to mutant (1+:3m) (Schuchert and Kohli, 1988). This suggests that the initiator acts as receiver of information and that disparity is probably due to loss of information in *cis* to an initiation site.

In *Ascobolus*, extreme disparities are also observed. In the progeny of wild-type/mutant crosses, putative single bp insertion mutations give an excess of conversion towards the mutant, whereas 1 bp deletion mutants give an excess towards wild-type. Studies in the *b2* ascospore color locus of *Ascobolus* imply that this disparity results from heteroduplex formation, followed by mismatch correction involving preferential excision of the shorter strand (Leblon and Rossignol 1973 ;

Leblon, 1979). Hastings et al. (1980) tested frame shift mutants in *Ascobolus* for parity in conversion versus restoration. Their results show near parity in conversion versus restoration. Conversion of a large deletion mutation in b2 exhibits strict parity in the direction of conversion. In the G234 deletion, conversions and restorations were measured. The genotype of the donor strand in the heteroduplex is preferentially recovered, irrespective of its G234 or wild-type nature (Nicolas and Rossignol, 1989). That is, they observe an excess of conversion over restoration. Some base substitution mutations also exhibit parity. In *Ascobolus*, both parity and disparity are seen in the b2 locus suggesting that this property is allele-specific.

E. Conversion tracts

The length of gene conversion tracts in yeast was estimated by the distance between two alleles included in the co-conversion event in heteroallelic crosses. Meiotic gene conversion tracts are usually continuous. In the *ARG4* locus, the tracts span a few hundred base pairs (Fogel and Mortimer, 1969), and in the *SUP6* locus tracts extend to several kilobases and sometimes greater than 14 centimorgans (DiCaprio and Hastings, 1976). Studies using restriction site markers on yeast chromosome III show that although most conversion tracts have a minimal length of 1 to 2 kb, about one quarter of the tracts are more than 5 kb in length (Symington and Petes 1988). In a similar study, Borts and Haber (1987) observed that conversion tracts are usually in the range of 1 to 2 kb.

In *Ascobolus*, however, gene conversion tracts are sometimes discontinuous (Kalogeropoulos and Rossignol, 1980). Occasionally, a marker that showed 2:2 segregation was closely flanked by other sites that showed aberrant segregation patterns. Discontinuous tracts can be interpreted as independent repair of different mismatches within a heteroduplex (Kalogeropoulos and Rossignol, 1980). It should be

noted that estimations of co-conversion frequencies are based on the assumption that conversion and restoration are equally likely.

F. A low PMS allele co-converts a high PMS allele

There is much evidence to show that when a PMS allele is placed near a normal allele, the PMS allele is co-converted, taking on the conversion spectrum of the normal allele. In *Ascobolus*, Leblon and Rossignol (1973) reported this phenomenon. When a C type allele (showing high PMS) is placed near an A type (showing low PMS) the C type allele no longer shows PMS. It shows the conversion spectrum of the A type allele. The conversion spectrum of the A type allele is unaffected. This suggests that conversion tracts are continuous, and that conversion starts at the normal allele, covers the PMS allele and co-converts it. In yeast, this has been seen in *HIS1* and *ARG4*. The frequency of PMS at *his1-49* is reduced to 18% in the presence of *his1-1S*, which shows no PMS, whereas *his 1-49* shows 85% PMS when it is alone (Hastings, 1984). The PMS rate of *arg4-16* is reduced from 48% to 28% in the presence of *arg4-19* or *arg4*-17 (Fogel et al., 1979).

To summarize, the NMS frequency of an allele should depend on whether or not heteroduplex DNA is formed, whether heteroduplex is formed on one chromatid or two chromatids, whether the heteroduplex is repaired or not, and whether the repair is towards restoration or conversion.

Molecular mechanism of mismatch repair

A. Mismatch repair in prokaryotes

Mismatch repair studies have been done mainly on two bacteria: *Escherichia coli* and *Streptococcus pneumoniae* (Pneumococcus). Evidence for base mismatch repair in bacteria came from the transformation studies of *S. pneumoniae*. Transformation results from insertion of a single-strand of donor DNA into the recipient genome. Marker-specific variation in transforming efficiency was observed

in studies involving different mutations in the same locus (Ephrussi-Taylor, 1966; Lacks, 1966). Ephrussi-Taylor (1966) proposed that some single-site donor markers were removed by a repair process after integration into recipient DNA. This was supported by the isolation of mismatch repair-deficient mutants, termed *hex*, which no longer show marker effects when used as recipients (Lacks, 1970). Marker-specific variations were observed in *H. influenzae* also (Stuy, 1965). In phage, multiple factor crosses show that the frequency of multiple recombinants is higher than predicted from the product of single crossover frequencies (White and Fox, 1974). This is called localized negative interference. Mismatch repair of heterozygosities at the heteroduplex stage of recombination has been frequently invoked to explain these phenomena. It was suggested that high negative interference with certain markers in the phage crosses results from mismatch repair favouring a particular allele in the mismatch (Lieb, 1983). This type of repair involves excision of a small patch (which is discussed later). Similar observations have been made for *E. coli* chromosomal recombination after transduction (Stadler and Kariya, 1973) or conjugation (Norkin, 1970) and for interactions between chromosomes and episomes within partially diploid cells (Coulondre and Miller, 1977). The *hex* mutants in *S. pneumoniae* behave as mutator alleles (Tiraby and Fox, 1974). It was suggested that the mismatch repair system is involved in mutation avoidance by correcting replication errors and improving the fidelity of DNA replication. Mutants deficient in mismatch repair are mutators and exhibit increased spontaneous mutation rates. In *E. coli*, many mutator alleles have been identified, and a set of alleles, *mutL*, *mutS*, *mutH* and *mutU*, have been shown to affect mismatch repair (reviewed by Cox, 1976; Modrich, 1991). It is obvious that not all mutators are involved in mismatch repair. Direct evidence for mismatch repair comes from artificial systems. Studies on transfection with lambda heteroduplexes in *E. coli* showed heteroduplex correction and led to the identification of the *mutL*, *mutS*, and *mutU* genes (Rydberg, 1978). Mismatch repair has also been

reported in transfection assays of *H. influenzae* and *B. subtilis* (Bagci, 1982; Trautner and Spatz, 1973). A cell-free extract system in *E. coli* supporting mismatch correction *in vitro* has also shown the involvement of *mutLSHU* genes in repair (Lu et al., 1983). The above evidence suggests the prevalence of homologous mismatch repair systems in prokaryotes. The function of the repair systems is to recognize and repair mismatches.

Further studies of *S. pneumoniae* showed variation in transformation efficiencies. Some markers called high efficiency markers (HE) are transformed very efficiently and some are transformed with low efficiency (LE) (Claverys and Lacks, 1986). It was suggested that LE markers are mismatch repaired and eliminated from incorporation and HE markers are repaired very poorly and stay in the heteroduplex, resulting in a high efficiency of transformation. This indicated that donor DNA is prevented from integrating stably into the recipient chromosome. It was suggested that the mismatch repair system acts on the heteroduplex and excises the donor DNA. Marker effects are not seen in the *hex* mutants. In those mutants both LE and HE markers are transformed very efficiently. Transformation was done with double mutants involving LE and HE markers in *cis*. The transformation efficiency of HE markers decreased to the level of LE markers (Lacks, 1966). The extent to which the LE marker affects the HE marker depends inversely on the distance between them. The average length of the excluded segment is 3 kb (Lacks, 1977). This was interpreted to mean that LE markers are efficiently recognized by the *Hex* system and repaired, and that HE markers are co-repaired along with LE markers. This co-repair in Pneumoccous may be analogous to that seen in yeast and *Ascobolus*. LE markers are analogous to conversion alleles in which the repair is normal. HE markers are analogous to PMS alleles. Normal alleles of yeast co-convert high PMS alleles in the same way that the LE markers co-correct the HE alleles. It is not clear how the donor DNA is discriminated against the native strand in Pneumoccous. However, it was suggested that a single-strand nick may be involved in this process. If the transforming

DNA is irradiated with UV, the transformation efficiency goes down and there is no difference in *hex*- cells (Tiraby and Sicard, 1973).

In *S. pneumoniae*, all transition mutations are repaired efficiently and show low transformation efficiency (LE markers). Transversion mutations show intermediate efficiency except for C/C mismatches which escape correction and show high efficiency of transformation (Claverys et al., 1983). Deletions or insertions of 1 or 2 bp are repaired efficiently, whereas 3 bp deletions or insertions are only partially repaired. Deletions of 5 bp and above are poorly repaired (Gasc et al., 1987). In the *hex* system, two genes, *HexA* and *HexB*, are involved. Both are necessary for repair (Claverys and Lacks, 1986).

Parallel work has been done in *E. coli*. Due to the advantage of its powerful molecular biology system, many aspects of mismatch repair are characterized better in *E. coli* than in *S. pneumoniae*. However, in many respects they are identical. Transfection studies using lambda led to the identification of mismatch repair genes. Many mutants have been identified which are involved in mismatch repair in *E. coli*. Depending on the type of mismatch, two repair systems operate: (i) very short-patch mismatch repair (VSPMR) and (ii) long-patch mismatch repair (LPMR) (reviewed by Radman, 1988).

The VSPMR system recognizes and repairs those G/T mismatches that originate by deamination of 5-methyl cytosine to T in the sequence CCAGG. This is the recognition sequence of *E. coli* cytosine methylase. Such G/T mismatches are repaired exclusively to the G-C pair, using a very short excision-resysnthesis tract. In other organisms like *S. pneumoniae*, *H. influenzae* and *B. subtilis* it is not known whether this system exists.

The LPMR repair system of *E. coli* is methyl-directed. It requires either unmethylated 5'-GATC-3' sequences or nicks (single strand breaks). In order for an enzyme to be able to correct replication errors it must detect the mismatch, distinguish

newly synthesized strands from parental strands, and act selectively to preserve the parental sequence (reviewed by Modrich,1991). Because the newly synthesized strands are transiently undermethylated, mismatch repair occurs preferentially, if not exclusively, on the newly synthesized strand of the heteroduplex. When such heteroduplexes are unmethylated, mismatch repair works equally well on either strand. In *E.coli*, the *dam* gene product methylates the adenine of d(G-A-T-C). Mutants deficient in this gene *(dam⁻)* are hyper-mutable due to loss of strand bias in mismatch repair. The DNA molecules derived from these strains are unmethylated. Transfection of *dam⁺ E. coli* strains with heteroduplexes made from lambda DNA isolated from *dam⁻* mutants shows no strand bias in repair (Pukkila et al., 1983 and Dohet et al., 1985).

Although much is known about the *E.coli* mismatch repair system, many specific aspects of the mismatch mechanism are not yet understood. Different models have been proposed to account for the available information about mismatch repair. All the models account for the requirement of the four gene products encoded by *MutL, S, H and U.*

MutLSHU repair in *E.coli*

All four gene products are required for mismatch repair both *in vivo* and *in vitro*. The MutS protein is proposed to be involved in the recognition of the mismatch (Modrich, 1987). Foot-printing analysis has demonstrated that the MutS protein specifically binds to DNA regions containing single base-pair mismatches. MutL protein is also required for repair and this is also implicated to be involved early in the process, possibly in the recognition of mismatches by MutS protein. The hemimethylated GATC sequence on either side of a recognized mismatch is nicked on the unmethylated strand by the MutH protein (Modrich, 1987). The MutU protein (helicaseII) presumably melts the nicked strand, sometimes over several kilobases, to allow repair synthesis by DNA polymerase.

The MutLSHU system does not recognize and repair all mismatches with equal efficiency. The extent of repair depends on the nature of the mismatch and its sequence environment (Radman and Wagner, 1986). In general, it appears that transition mutation mismatches (G/T and A/C) are better repaired than transversion mutation mismatches. The efficiency also increases with increasing G+C content in the neighborhood of the mismatches (Jones et al., 1987). The *E. coli* mismatch repair system can recognize and repair heteroduplexes containing one-base deletion or addition frame shift mutations very efficiently (Dohet et al., 1986). *In vitro* studies showed that all eight mismatches located at the same position were not corrected with equal efficiency. G/T is corrected very efficiently, while A/C, C/T, A/A, T/T and G/G are repaired at rates from 40-80% of the G/T mispair. Correction of each of these six mispairs occurs in a methyl-directed manner in a reaction requiring *mutH*, *mutL* and *mutS* gene products. C/C is not corrected efficiently.

During repair, even for single base mismatches, a long stretch of DNA is excised and resynthesised, as in the *hex* system. Repair tracts in mismatched DNA heteroduplexes were studied by Wagner and Meselson (1976) by transfecting *E. coli* with *in vitro* heteroduplexed λ DNA. Repair involving two or more close sites in the same heteroduplex occurs more often on the same strand than on opposite strands, and the tract length averages about 3 kb. In an *E. coli in vitro* system, Fishel and Kolodner (1984) show co-repair of mismatches separated by up to 1243 bp. This suggests that excision-resynthesis tracts can cover several kb. Repair of symmetrically methylated DNA requires the *mutS* gene product and also requires the *recF* gene product. It was suggested that the un-methylated DNA sequence between two GATC sequence with a mismatch is excised and resynthesised (reviewed by Modrich, 1987).

B. Mismatch repair in eukaryotes

In eukaroytes, much of the information about mismatch repair comes from yeast. In yeast (*S. cerevistae*), like in *E. coli*, the long patch repair system seems to operate in recombination. Though not much molecular data are available, the genetic data provide evidence that recombinational repair is long and continuous. The following review will show that this may be similar to the *E. coli* system in many respects.

Mutants at the *PMS1* locus of yeast exhibit increased PMS frequencies among unselected tetrads (Williamson et al., 1985). In addition, they display a mitotic mutator phenotype. These phenotypes suggest that the *PMS1* gene product is involved in DNA mismatch repair. *PMS2* and *PMS3* yield similar phenotypes but their effects on DNA mismatch have not been examined (Williamson et al., 1985). The *PMS1* gene was cloned and sequenced (Kramer et al., 1989). The deduced amino acid sequence of the PMS1 protein exhibited homology to those of the *mutL* gene product from *Salmonella typhimurium* and the *hexB* gene product from *Streptococcus pneumoniae*, proteins required for DNA mismatch repair in those organisms.

Kramer et al. (1989) constructed heteroduplex DNA with the *ADE8* gene *in vitro*, on a centromeric plasmid (YCp50) which is expected to maintain a single copy, and transformed yeast cells. For construction of heteroduplex DNA, plasmids were chosen such that one carried a functional *ADE8* allele and the other carried a defective *ade8* allele. Heteroduplex DNAs were constructed from linearized double-stranded DNA of one plasmid and circular single-stranded DNA of a second plasmid differing from the first by a defined structural change. Repair *in vivo* can be monitored by the color of the colony. Unrepaired heteroduplex results in sectored red and white colonies. Their studies showed that the efficiency of repair is dependent on the type of mismatch. Single nucleotide additions and deletions are repaired efficiently and substitution mismatches are repaired with high to intermediate efficiencies. Among the

mismatches, C/C and 38 nucleotide loop with *ade8-18/ADE8* were corrected with low efficiency. Repair of these mismatches was severely impaired in the putative DNA mismatch repair mutants *pms1*, *pms2* and *pms3*. Lichten et al. (1990) developed a special gel electrophoresis technique to detect the mismatches in the duplex DNA. Gels were run at high voltage and high temperature so that the duplexes with a mismatch will melt and run as a single stranded DNA which can be detected by probing. At the appropriate temperature, C-C and G-G mismatches can be resolved both from one another and from the two parental homoduplexes. By using this technique, they showed that only the C/C mismatch is repaired poorly during meiosis. Detloff et al. (1991) developed a genetic method for identifying which specific mismatch pair is left unrepaired in PMS events. They showed that C/C mismatches were repaired inefficiently relative to all other point mismatches. The other mismatches (G/G, G/A, T/T, A/A, T/C, C/A, and T/G) were repaired with approximately the same efficiency.

Bishop et al. (1989) transformed *S. cerevisiae* with *in vitro* constructed heteroduplex plasmid DNA. Heteroduplex correction was monitored by the presence or absence of β-galactosidase activity. Repair efficiency varied with the type of heteroduplex. A/C, G/T, G/A and G/G mismatches were repaired with greater efficiencies than C/C, A/A, T/T and TGA/GAA. The mismatch T/C was repaired with an intermediate efficiency. The efficiency of repair was reduced in *pms1-1* cells compared with wild-type cells.

Some work has been done with mammalian cells using heteroduplex transfection. Brown and Jiricny (1988) transfected monkey kidney cells with SV40 DNA molecules harbouring single base mismatches. All the mismatches were corrected with different efficiencies. The mispairs G/T, A/C, C/T, and A/G were corrected with efficiencies of 96%, 78%, 72% and 39%, respectively. The repair efficiency of homogeneous mispairs G/G, C/C, A/A and T/T were corrected with 92%, 66%, 58% and 39% efficiencies, respectively.

In vitro mismatch repair in cell-free extracts

Heteroduplex DNAs containing 4 or 7 base pair insertion or deletion mismatches, or each of the eight possible substitution mismatches were constructed by Muster-Nassal and Kolodner (1986). Extracts of yeast mitotic cells catalyzed the correction of the mismatched nucleotides. They showed that insertion/deletion mismatches and A/C and G/T mismatches are repaired efficiently, while the six other single base-pair mismatches are repaired poorly or at undetectable rates. Mismatch correction in yeast requires the *PMS* genes. Correction was accompanied by incorporation of less than 20 nucleotides at or near the site of the repaired mismatch.

The *arg4-16/ARG4* allele pair in yeast can form G/G or C/C mismatches and display 33% PMS in wild-type and 60% in *pms1* mutants (Williamson et al., 1985). This is interpreted to indicate that the higher PMS values in *pms1* mutant strains reflect the efficiency of repair of mismatches in the heteroduplex. A comparison was made between PMS and repair efficiencies of different types of mismatches (Kramer et al., 1989). Their results suggest that C/C mismatches are repaired less efficiently and show high level of PMS.

As discussed earlier, in yeast, large insertions (>100-bp) and deletions have normal rates of gene conversion, and do not have high rates of PMS (Fink and Styles, 1974). The *ade8-18/ADE8* allele pair can form heteroduplex DNA with a 38 nucleotide loop during meiosis and shows a high frequency of PMS (>50%) (Fogel et al., 1981). Kramer et al. (1989) showed that heteroduplex DNA made *in vitro* with *ade8-18* and *ADE8* is not corrected efficiently after transformation. In mammalian cells, Weiss and Wilson (1987) constructed 13 different heteroduplexes from SV40 wild-type and deletion mutant DNAs, each heteroduplex contained one or multiple single-stranded loops in the intron of the gene for large tumor antigen. After transfection into cultured monkey cells, cellular repair evaluation showed that single-stranded loops were corrected with

an overall efficiency of 90%. Repair of single-stranded loops was biased nearly 2 to 1 against the loop. The efficiency, accuracy, and the strand bias of repair were unaffected by loop size within the tested range, which was 25-247 nucleotides. The excision tract with repair of single-stranded loops rarely exceeds 200-400 nucleotides in length. Repair of palindromic insertions in heteroduplex was not tested *in vitro*, but *in vivo* repair studies in yeast based on PMS showed that they are corrected inefficiently (Nag et al., 1989).

6. Significance of mismatch repair

Apart from repairing the mismatches formed during recombination or replication, mismatch repair systems have an important role to play in preventing homeologous recombination. The hybrid joint is a key structure for aligning complementary DNA strands, one contributed by each of the recombining molecules (Holliday, 1964). Recombination *in vivo* requires nearly perfect homology and is abolished by as little as 10-20% sequence divergence (Schneider et al., 1981 and Shen and Huang, 1986), presumably to maintain chromosomal integrity. However, heteroduplex DNA formation catalyzed *in vitro* by the *E. coli* RecA protein tolerates up to 30% sequence mismatches (Das Gupta and Radding, 1982). Studies on recombination and mismatch repair in *Streptococcus pneumoniae* (Claverys and Lacks 1986) and *E. coli* (Jones et al., 1987, and Feinstein and Low, 1986) suggested to Radman (1988) that the marker effects on the efficiency of homologous recombination are often caused by the abortion of heteroduplex intermediates with one or few mismatches. Thus the mismatch repair system might prevent recombination between partially homologous sequences and provide a functional barrier to interspecies recombination (Radman, 1988). Mutations in the *mutL*, *mutS* or *mutH* mutator genes can increase intergeneric recombination. In these mutants intergeneric recombination occurs more efficiently

between *E. coli* and *Salmonella typhimurium* which are ~20% divergent in DNA sequence (Rayssiguier et al., 1989).

7. Other genes involved in mismatch repair

Studies of mutations that affect DNA metabolism in *E. coli* have shown that DNA replication, repair and recombination have functional overlap in the utilization of common enzymes (reviewed by Smith, 1988) which may reflect the use of similar substrates. Mutants that affect DNA replication and repair often show decreased or increased recombination rates. Many recombination mutants have been isolated in *E. coli*. They include DNA binding proteins, a strand transferase (RecA protein), topoisomerases, ligases, DNA polymerases, endo- and exo-nucleases and resolvases.

As in *E. coli*, many repair genes in yeast are also involved in DNA metabolism. In yeast, these mutants are loosely grouped into three different classes. The first group is from the *cdc* (cell division cycle) mutants. Hyper-recombination mutants have been found among the *cdc* mutants, especially those that arrest in the S or G2 phase of the cell cycle. These include mutations in the *CDC2* (DNA polymeraseII), *CDC6*, *CDC9* (DNA ligase), *CDC13* and *CDC 17* (DNA polymerasel) genes (reviewed by Smith, 1988). *Cdc5* and *cdc14* mutants, which arrest the cells in G2, also have been reported to be hyper-recombinogenic. The second group comes from the *rad* collection of mutants. They define three epistasis groups *RAD3*, *RAD6* and *RAD50*. The *RAD3* group is required for excision repair of lesions in DNA. It includes *RAD1* and *RAD4*. The *RAD6* group is involved in error-prone or mutagenic repair. *RAD6*, *RAD9* and *RAD18* are in this pathway. The third epistasis group is recombinational repair which includes *RAD50* and *RAD52* (reviewed by Friedberg, 1988). The third group is specific to meiosis and includes *SPO11*, (Esposito and Esposito, 1969) *SPO13*, (Klapholz and Esposito, 1980) *HOP1*, (Hollingsworth and Byers, 1989) *MEI4* (Menees and Roeder, 1989) and *MER1* (Engebrecht and Roeder, 1989).

As seen above, the recombination process is complex involving many genes. For simplicity, the recombination steps can be viewed as a pathway which involves initiation, formation of the intermediate, processing of the intermediate and resolution of the intermediate. A defect in any of these steps will affect the outcome of recombination. Conversely, a defect in any of these steps can be inferred from the outcome of the recombination. As stated in the introduction this project is designed to understand the mismatch repair process, which is involved in the processing of intermediates, by studying the outcome of recombination.

MATERIALS AND METHODS

MATERIALS

1. Chemicals and enzymes

Chemicals used in this study were obtained from Sigma, DIFCO or BBL. All the restriction and modifying enzymes were purchased either from BRL or from Pharmacia. Some of the enzymes and reagents were supplied with a kit. For dissection of spores, Glusulase (β-Glucuronidase) was purchased from Sigma. Zymolyase (20T) was supplied by ICN Immuno Biologicals, CA. The Gene Clean kit was obtained from BIO/CAN Scientific, CA. The random primer radio-labeling kit was from BRL and the sequencing kit was from US Biochemicals, Ohio. The Mutagene kit for *in vitro* mutagenesis was obtained from Bio-Rad laboratories. 5 Fluoro-orotic acid (FOA) was purchased from PCR Inc, Florida.

2. Media

A. *E. coli*

All the *E. coli* strains were grown in standard LB medium containing 10 g tryptone, 10 g sodium chloride and 5 g yeast extract in one liter of water. Twelve grams of Bactoagar was added for plates (Maniatis et al., 1982). For the isolation of single-stranded DNA 2x YT medium containing 16 g Bactotryptone, 10 g yeast extract and 5 g NaCl in a liter of water was used instead of LB medium.

B. Yeast

Media used for yeast cultivation are:

YEPD (Yeast Extract, Peptone, Dextrose): 1% bacto-yeast extract, 2% peptone, 2% dextrose in distilled water.

MC (Mortimer Complete): 0.67% bacto-yeast nitrogen base without amino acids and 2% glucose in distilled water, and 10 ml of amino acid stock solution per liter added after autoclaving. The stock solution contained 20 mg each of adenine, arginine, histidine, lysine, methionine, tryptophan and uracil, 30 mg of leucine and 350 mg of threonine in 100 ml.

YPA (pre-sporulation medium): 2% potassium acetate (wt/vol), 2% peptone and 1% yeast extract in distilled water.

Sporulation medium: 1% potassium acetate, 0.1% yeast extract and 0.05% dextrose in distilled water.

Omission media: MC medium lacking one or more of the components of the stock solution. For example, MC-his represents omission of histidine.
For plates 2% agar was added before autoclaving.

FOA (5-fluoro-orotic acid) plates : 1.7 g of yeast nitrogen base without amino acids, 5 g ammonium sulphate, 20 g dextrose and 20 g agar were mixed in 800 ml water and autoclaved. In a separate flask, 1 g of 5-fluoro-orotic acid powder was dissolved in 200 ml lukewarm water and filter sterilized. After autoclaving, the first mix was cooled to 45°C. Both solutions were mixed and 10 ml of amino acid complete medium stock solution was added before pouring onto the plates.

3. Plasmids and Strains

A. Plasmids

PTZ18: This is a derivative of a PUC plasmid. In addition to a colE1 replication origin, it carries the gene coding for ampicillin resistance and the *lacZ* gene fragment of the β-galactosidase gene from *E. coli*

and the f1 phage IG region which makes it possible to generate the single-stranded DNA from this plasmid by superinfecting with appropriate helper phages (Norander et al 1983). The size of the plasmid is 2.86 kb. This was supplied with the Mutagene Phagemid kit and used for general cloning and isolation of single stranded DNA for *in vitro* mutagenesis.

YCp50: This is the source of the *URA3* gene. The *URA3* fragment (1.9 kb) can be liberated by restricting with *Sall* and *Sma*I (Rose et al., 1987). This 1.9 kb *URA3* fragment was used further for disruption of the *HIS1* gene.

pHIS1: This plasmid contains the *HIS1* gene inserted into the *Bam*HI and *Eco*RI sites of YCp50. This plasmid was provided by Dr. Elizabeth Savage.

There are 11 plasmids (from pHP1 to pHP11) which were constructed for this study and the details of construction are explained in the Methods section.

B. *E. coli* strains

CJ236:

dut, ung, thi, relA; pCJ105 (Cm^r). The *dut* and *ung* phenotypes result in substitution of uracil for thymine on the DNA. This strain was used to produce single-stranded DNA for *in vitro* mutagenesis from pHP1. The F' plasmid pCJ105 carries information for pili construction. The pili are necessary for both phagemid and helper phage attachment to and entry into the bacterial cells (Kunkel et al. 1987). This was also supplied with the phagemid kit.

MV1190:

Δ(lac-proAB), thi, supE, (Δ(srlR-recA) 306::Tn10 (tet^r) [F':tra D36, pro AB, lac I^q ZΔM15] (Messing 1983). This was used to transform double-stranded DNA after synthesizing the complementary strand having a mutation during *in vitro* mutagenesis. As this strain is *ung*$^+$ the phagemid strands containing uracil instead of T are selectively eliminated, leaving the mutated strand. This strain was supplied with the Mutagene kit.

DH5α:

F' φ 80d *lacZ* Δ M15 Δ *(lacZ* YA-*argF)* U169 *recA1 endA1 hsdR17* (r_k^-, m_k^+) *supE44* λ$^-$thi-1 *gyA relA1.* (Hanahan, 1983). The competent cells were obtained form BRL. They were used as a host for the propagation of plasmid DNA.

M13K07 helper phage:

This was derived from an M13 mutant phage in which DNA replication has been partially disabled by insertion of DNA carrying the kanamycin resistance gene. This disability and the high copy number of phagemids lead to packaging of the phagemid at the expense of the helper phage. The presence of kanamycin selects for the cells infected with helper phage (Vieria and Messing, 1987).

C. Yeast strains

The list of yeast strains is presented in Tables 1 and 2. The haploids are presented in Table 1 and the diploids in Table 2. All the diploid strains were made for this study. The diploids heterozygous at *HIS1* were used to study the frequency of PMS for the *his1* alleles. All other *his1* heteroallelic diploids were used to study both mitotic and meiotic intragenic recombination at the *HIS1* locus.

The following are the sequence changes in the pre-existing *his1* alleles (Savage et al., 1989) that were used in this study:

HIS1 allele	Change	Position in the gene
his1-1	A to T	+208
his1-S	A to T	+364
his1-19	A to G	+432
his1-42	G to A	+499
his1-30	G to A	+652
his1-51	G to A	+653
his1-40	G to A	+683
his1-7	G to A	+798
his1-315	G to A	+887
his1-49	37 bp deletion	from +163 to 200

Table 1. Haploid strains used in this study

Strain	Genotype
LZ21-1C*	*MATα ura3 leu2*
LZ21-13C*	*MATα ura3 hom3 arg6 trp2 leu2*
HP109-1C*	*MATα hom3* ***his1-1*** *ade2*
HP109-3B*	*MATα* ***his1-1*** *ade2*
HP109-8D*	*MATα hom3* ***his1-1*** *ade2*
LZ24-4B*	*MATα* ***his1-1S*** *ade2*
LZ24-8A*	*MATα* ***his1-1S*** *ade2*
LZ24-11D*	*MATα* ***his1-1S*** *arg6 ade2*
HP119-11*C	*MATα* ***his1-7*** *ade2*
HP119-8B*	*MATα* ***his1-7*** *leu2 ade2*
PM5-3B**	*MATα* ***his1-7*** *arg6 leu2 ade2*
HPO17-3D*	*MATα* ***his1-19*** *ade2*
HP017-8A*	*MATα* ***his1-19*** *ade2*
LZ10-2A*	*MATα ura3* ***his1-30*** *trp2 ade2*
LZ10-8B*	*MATα ura3* ***his1-30*** *ade2*
PM2-3B**	*MATα* ***his1-30*** *arg6ade2*
PM2-4B**	*MATαhom3* ***his1-30*** *ade2*

.....Continued

Table 1. Continued

Strain	Genotype
HP077-1D*	*MATα ura3* ***his1-40*** *ade2*
HP077-2B*	*MATaura3* ***his1-40*** *ade2*
PM3-3D**	*MAT α ura3* ***his1-40*** *arg6 ade2*
PM3-8C**	*MATα hom3* ***his1-40***
HP067-3A*	*MATα* ***his1-42*** *ade2*
HP067-4B*	*MATa* ***his1-42*** *ade2*
LZ21-1B*	*MATa hom3* ***his1-49*** *arg6 trp2 leu2*
LZ21-2A*	*MAT a ura3* ***his1-49*** *leu2*
PM1-13**	*MATα* ***his1-49*** *arg6 ade2*
PM1-19**	*MATα ura3 hom3* ***his1-49*** *arg6 trp2 leu2*
HP019-1A*	*MATα* ***his1-51*** *ade2*
HP019-1C*	*MATa****his1-51*** *ade2*
HP007-3B*	*MATa* ***his1-315*** *leu2 ade2*
HP007-9A*	*MATα* ***his1-315*** *trp2 ade2*

.....Continued

Table 1. continued

Strain	Genotype
PM21**	*MATα ura3 hom3* ***his1::URA3*** *arg6 trp2 leu2* (*HIS1* disruption at *Sal*I site in LZ21-13C)
PM22**	*MATα ura3 hom3* ***his1::URA3*** *arg6 trp2 leu2* (*HIS1* disruption at *Eco*RV site in LZ21-13C)
PM23**	*MATα ura3 hom3* ***his1-260*** *arg6 trp2 leu2* (*Sal*I site fill-in = gene replacement in PM21))
PM24**	*MATα ura3 hom3* ***his1-511*** *arg6 trp2 leu2* (*Bgl*II site fill-in = gene replacement in PM21)
PM25**	*MATα ura3 hom3* ***his1-216*** *arg6 trp2 leu2* (gene replacement in PM21)
PM26**	*MATα ura3 hom3* ***HIS1-258*** *arg6 trp2 leu2* (gene replacement in PM1-19)
PM27**	*MATα ura3 hom3* ***his1-662*** *arg6 trp2 leu2* (gene replacement in PM22)
PM28**	*MATα ura3 hom3* ***his1-672*** *arg6 trp2 leu2* (gene replacement in PM22)
PM29**	*MATα ura3 hom3* ***his1-876*** *arg6 trp2 leu2* (gene replacement in PM22)

.....Continued

Table 1. continued

Strain	Genotype
PM31**	*MATα ura3 hom3* ***his1-258::URA3*** *arg6 trp2 leu2* (*HIS1-258* disruption at *Eco*RV site in PM26)
PM32**	*MATa ura3* ***his1-40::URA3*** *ade2* (*his1-40* disruption at *Sal*I site in HP077-2B)
PM33**	*MATa ura3* ***his1-30::URA3*** *trp2 ade2* (*his1-30* disruption at *Sal*I site in LZ10-2A)
PM34**	*MATa ura3* ***his1-25840*** *ade2* (gene replacement in PM32)
PM35**	*MATa ura3* ***his1-25830*** *trp2 ade2* (gene replacement in PM33)
PM36**	*Mat α ura3 hom3* ***his1-258672*** *arg6 trp2leu2* (gene replacement in PM31)
PM115-12D	*MATa* ***his1-216*** *trp2 ade2*
PM117-8C	*MATa ura3* ***his1-672*** *ade2*

* Strains from this laboratory.

** Strains made for this study.

Table 2. Diploid strains used in this study

Diploid	Haploid parents	Genotype
PM101	LZ21-1B	*MATa* *hom3* **his1-49** *arg6* *trp2* *leu2* *ADE2*
	LZ24-8A	*MATα* *HOM3* **his1-1S** *ARG6* *TRP2* *LEU2* *ade2*
PM102	LZ21-1B	*MATa* *hom3* **his1-49** *arg6* *trp2* *leu2* *ADE2*
	HPO17-8A	*MATα* *HOM3* **his1-19** *ARG6* *TRP2* *LEU2* *ade2*
PM103	LZ21-1B	*MATa* *hom3* **his1-49** *arg6* *trp2* *leu2* *ADE2*
	HPO67-3A	*MATα* *HOM3* **his1-42** *ARG6* *TRP2* *LEU2* *ade2*
PM104	LZ21-1B	*MATa* *URA3* *hom3* **his1-49** *arg6* *trp2* *leu2* *ADE2*
	LZ10-2A	*MATα* *ura3* *HOM3* **his1-30** *ARG6* *TRP2* *LEU2* *ade2*
PM105	LZ21-1B	*MATa* *hom3* **his1-49** *arg6* *trp2* *leu2* *ADE2*
	HPO19-1A	*MATα* *HOM3* **his1-51** *ARG6* *TRP2* *LEU2* *ade2*
PM106	LZ21-1B	*MATa* *URA3* *hom3* **his1-49** *arg6* *trp2* *leu2* *ADE2*
	HPO77-1D	*MATα* *ura3* *HOM3* **his1-40** *ARG6* *TRP2* *LEU2* *ade2*
PM107	LZ21-1B	*MATa* *hom3* **his1-49** *arg6* *trp2* *leu2* *ADE2*
	HP119-8B	*MATα* *HOM3* **his1-7** *ARG6* *TRP2* *LEU2* *ade2*
PM108	LZ21-1B	*MATa* *hom3* **his1-49** *arg6* *trp2* *leu2* *ADE2*
	HPOO7-9A	*MATα* *HOM3* **his1-315** *ARG6* *trp2* *LEU2* *ade2*
PM109	LZ21-2A	*MATa* *ura3* *HOM3* **his1-49** *ARG6* *TRP2* *leu2*
	PM23	*MATα* *ura3* *hom3* **his1-260** *arg6* *trp2* *leu2*

.....continued

Table 2. Continued

Diploid	Haploid parents		Genotype					
PM110	LZ21-2A	*MATa*	*ura3*	*HOM3*	***his1-49***	*ARG6*	*TRP2*	*leu2*
	PM24	*MATα*	*ura3*	*hom3*	***his1-511***	*arg6*	*trp2*	*leu2*
PM112	LZ21-2A	*MATa*	*ura3*	*HOM3*	***his1-49***	*ARG6*	*TRP2*	*leu2*
	PM27	*MATα*	*ura3*	*hom3*	***his1-662***	*arg6*	*trp2*	*leu2*
PM115	LZ21-1C	*MATa*	*ura3*	*HOM3*	***HIS1***	*ARG6*	*TRP2*	*leu2*
	PM25	*MATα*	*ura3*	*hom3*	***his1-216***	*arg6*	*trp2*	*leu2*
PM116	LZ21-1C	*MATa*	*ura3*	*HOM3*	***HIS1***	*ARG6*	*TRP2*	*leu2*
	PM27	*MATα*	*ura3*	*hom3*	***his1-662***	*arg6*	*trp2*	*leu2*
PM117	LZ21-1C	*MATa*	*ura3*	*HOM3*	***HIS1***	*ARG6*	*TRP2*	*leu2*
	PM28	*MATα*	*ura3*	*hom3*	***his1-672***	*arg6*	*trp2*	*leu2*
PM118	LZ21-1C	*MATa*	*ura3*	*HOM3*	***HIS1***	*ARG6*	*TRP2*	*leu2*
	PM29	*MATα*	*ura3*	*hom3*	***his1-876***	*arg6*	*trp2*	*leu2*
PM119	LZ21-1C	*MATa*	*ura3*	*HOM3*	***HIS1***	*ARG6*	*TRP2*	*leu2*
	PM27	*MATα*	*ura3*	*hom3*	***his1-662***	*arg6*	*trp2*	*leu2*
PM120	HP109-1C	*MATa*	*hom3*	***his1-1***	*ARG6*	*ade2*		
	PM1-13	*MATα*	*HOM3*	***his1-49***	*arg6*	*ade2*		
PM121	HP109-1C	*MATa*	*URA3*	*hom3*	***his1-1***	*ARG6*	*ade2*	
	PM3-3D	*MATα*	*ura3*	*HOM3*	***his1-40***	*arg6*	*ade2*	
PM122	PM5-3B	*MATa*	*HOM3*	***his1-7***	*arg6*	*ADE2*		
	HP109-8D	*MATα*	*hom3*	***his1-1***	*ARG6*	*ade2*		

.....Continued

Table 2. Continued

Diploid	Haploid parents	Genotype
PM123	HP109-1C	*MATa* *hom3* ***his1-1*** *ARG6* *ADE2*
	PM23	*MATα* *HOM3* ***his1-1S*** *arg6* *ade2*
PM124	PM2-3B	*MATa* *HOM3* ***his1-30*** *arg6* *ade2*
	HP109-8D	*MATα* *hom3* ***his1-1*** *ARG6* *ADE2*
PM129	HP109-3B	*MATa* *URA3* *HOM3* ***his1-1*** *ARG6* *TRP2* *leu2*
	PM24	*MATα* *ura3* *hom3* ***his1-511*** *arg6* *trp2* *leu2*
PM130	HP109-3B	*MATa* *URA3* *HOM3* ***his1-1*** *ARG6* *TRP2* *leu2* *ade2*
	PM25	*MATα* *ura3* *hom3* ***his1-216*** *arg6* *trp2* *leu2* *ADE2*
PM131	LZ21-2A	*MATa* *ura3* *HOM3* ***his1-49*** *ARG6* *TRP2* *leu2*
	PM25	*MATα* *ura3* *hom3* ***his1-216*** *arg6* *trp2* *leu2*
PM132	LZ24-4B	*MATa* *URA3* *HOM3* ***his1-1S*** *ARG6* *TRP2* *LEU2* *ade2*
	PM25	*MATα* *ura3* *hom3* ***his1-216*** *arg6* *trp2* *leu2* *ADE2*
PM133	HP017-3D	*MATa* *URA3* *HOM3* ***his1-19*** *ARG6* *TRP2* *LEU2* *ade2*
	PM25	*MATα* *ura3* *hom3* ***his1-216*** *arg6* *trp2* *leu2* *ADE2*
PM134	HP067-4B	*MATa* *URA3* *HOM3* ***his1-42*** *ARG6* *TRP2* *LEU2* *ade2*
	PM25	*MATα* *ura3* *hom3* ***his1-216*** *arg6* *trp2* *leu2* *ADE2*
PM135	LZ10-8B	*MATa* *ura3* *HOM3* ***his1-30*** *ARG6* *TRP2* *LEU2* *ade2*
	PM25	*MATα* *ura3* *hom3* ***his1-216*** *arg6* *trp2* *leu2* *ADE2*

.....Continued

Table 2. Continued

Diploid	Haploid parents		Genotype
PM136	HP019-1C	*MATa*	*URA3 HOM3* **his1-51** *ARG6 TRP2 LEU2 ade2*
	PM25	*MATα*	*ura3 hom3* **his1-216** *arg6 trp2 leu2 ADE2*
PM137	HP119-11C	*MATa*	*URA3 HOM3* **his1-7** *ARG6 TRP2 LEU2 ade2*
	PM25	*MATα*	*ura3 hom3* **his1-216** *arg6 trp2 leu2 ADE2*
PM138	HP007-3B	*MATa*	*URA3 HOM3* **his1-315** *ARG6 TRP2 leu2 ade2*
	PM25	*MATα*	*ura3 hom3* **his1-216** *arg6 trp2 leu2 ADE2*
PM139	HP007-2B	*MATa*	*ura3 HOM3* **his1-40** *ARG6 TRP2 LEU2 ade2*
	PM25	*MATα*	*ura3 hom3* **his1-216** *arg6 trp2 leu2 ADE2*
PM140	PM115-12D	*MATa*	*URA3 HOM3* **his1-216** *ARG6 TRP2 LEU2 ade2*
	PM23	*MATα*	*ura3 hom3* **his1-260** *arg6 trp2 leu2 ADE2*
PM141	PM115-12D	*MATa*	*URA3 HOM3* **his1-216** *ARG6 TRP2 LEU2 ade2*
	PM24	*MATα*	*ura3 hom3* **his1-511** *arg6 trp2 leu2 ADE2*
PM142	PM115-12D	*MATa*	*URA3 HOM3* **his1-216** *ARG6 TRP2 LEU2 ade2*
	PM28	*MATα*	*ura3 hom3* **his1-672** *arg6 trp2 leu2 ADE2*
PM150	HP007-2B	*MATa*	*ura3 HOM3* **his1-40** *ARG6 TRP2 LEU2 ade2*
	PM28	*MATα*	*ura3 hom3* **his1-672** *arg6 trp2 leu2 ADE2*
PM151	HP019-1C	*MATa*	*URA3 HOM3* **his1-51** *ARG6 TRP2 LEU2 ade2*
	PM28	*MATα*	*ura3 hom3* **his1-672** *arg6 trp2 leu2 ADE2*

.....Continued

Table 2. Continued

Diploid	Haploid parents	Genotype
PM152	LZ10-8B	*MATa* *ura3* *HOM3* **his1-30** *ARG6* *TRP2* *LEU2* *ade2*
	PM28	*MATα* *ura3* *hom3* **his1-672** *arg6* *trp2* *leu2* *ADE2*
PM153	HP119-11C	*MATa* *URA3* *HOM3* **his1-7** *ARG6* *TRP2* *LEU2* *ade2*
	PM28	*MATα* *ura3* *hom3* **his1-672** *arg6* *trp2* *leu2* *ADE2*
PM154	HP007-3B	*MATa* *URA3* *HOM3* **his1-315** *ARG6* *TRP2* *leu2* *ade2*
	PM28	*MATα* *ura3* *hom3* **his1-672** *arg6* *trp2* *leu2* *ADE2*
PM155	HP109-3B	*MATa* *URA3* *HOM3* **his1-1** *ARG6* *TRP2* *leu2*
	PM28	*MATα* *ura3* *hom3* **his1-672** *arg6* *trp2* *leu2*
PM157	LZ21-2A	*MATa* *ura3* *HOM3* **his1-49** *ARG6* *TRP2* *leu2* *ade2*
	PM28	*MATα* *ura3* *hom3* **his1-672** *arg6* *trp2* *leu2* *ADE2*
PM170	PM5-3B	*MATa* *HOM3* **his1-7** *arg6* *ADE2*
	PM2-4B	*MATα* *hom3* *his1-30* *ARG6* *ade2*
PM171	PM5-3B	*MATa* *HOM3* **his1-7** *arg6* *ADE2*
	PM3-8C	*MATα* *hom3* **his1-40** *ARG6* *ade2*
PM173	PM3-3D	*MATa* *ura3* *HOM3* **his1-40** *arg6* *ade2*
	PM2-4B	*MATα* *URA3* *hom3* **his1-30** *ARG6* *ade2*
PM175	HP119-11C	*MATa* *URA3* *HOM3* **his1-7** *ARG6* *TRP2* *LEU2* *ade2*
	PM24	*MATα* *ura3* *hom3* **his1-511** *arg6* *trp2* *leu2* *ADE2*

.....Continued

Table 2. Continued

Diploid	Haploid parents		Genotype
PM178	HP067-4B	*MATa*	*URA3 HOM3* **his1-42** *ARG6 TRP2 LEU2 ade2*
	PM24	*MATα*	*ura3 hom3* **his1-511** *arg6 trp2 leu2 ADE2*
PM179	HP017-3D	*MATa*	*URA3 HOM3* **his1-19** *ARG6 TRP2 LEU2 ade2*
	PM24	*MATα*	*ura3 hom3* **his1-511** *arg6 trp2 leu2 ADE2*
PM180	HP007-3B	*MATa*	*URA3 HOM3* **his1-315** *ARG6 TRP2 leu2 ade2*
	HPY29	*MATα*	*ura3 hom3* **his1-876** *arg6 trp2 leu2 ADE2*
PM181	HP119-11C	*MATa*	*URA3 HOM3* **his1-7** *ARG6 TRP2 LEU2 ade2*
	PM29	*MATα*	*ura3 hom3* **his1-876** *arg6 trp2 leu2 ADE2*
PM182	HP077-2B	*MATa*	*ura3 HOM3* **his1-40** *ARG6 TRP2 LEU2 ade2*
	PM29	*MATα*	*ura3 hom3* **his1-876** *arg6 trp2 leu2 ADE2*
PM183	HP067-4B	*MATa*	*URA3 HOM3* **his1-42** *ARG6 TRP2 LEU2 ade2*
	PM29	*MATα*	*ura3 hom3* **his1-876** *arg6 trp2 leu2 ADE2*
PM184	LZ10-8B	*MATa*	*ura3 HOM3* **his1-30** *ARG6 TRP2 LEU2 ade2*
	PM29	*MATα*	*ura3 hom3* **his1-876** *arg6 trp2 leu2 ADE2*
PM185	LZ21-2A	*MATa*	*ura3 HOM3* **his1-49** *ARG6 TRP2 leu2 ade2*
	PM29	*MATα*	*ura3 hom3* **his1-876** *arg6 trp2 leu2 ADE2*
PM186	PM117-8C	*MATa*	*ura3 HOM3* **his1-672** *ARG6 TRP2 LEU2 ade2*
	PM29	*MATα*	*ura3 hom3* **his1-876** *arg6 trp2 leu2 ADE2*
PM187	PM115-2D	*MATa*	*URA3 HOM3* **his1-216** *ARG6 TRP2 LEU2 ade2*
	PM29	*MATα*	*ura3 hom3* **his1-876** *arg6 trp2 leu2 ADE2*

.....Continued

Table 2. Continued

Diploid	Haploid parents		Genotype
PM189	HP109-3B	*MATa*	*URA3 HOM3* **his1-1** *ARG6 TRP2 leu2 ade2*
	PM29	*MATα*	*ura3 hom3* **his1-876** *arg6 trp2 leu2* ADE2
PM190	HP077-2B	*MATa*	*ura3 HOM3* **his1-40** *ARG6 TRP2 LEU2 ade2*
	PM27	*MATα*	*ura3 hom3* **his1-662** *arg6 trp2 leu2* ADE2
PM191	HP019-1C	*MATa*	*URA3 HOM3* **his1-51** *ARG6 TRP2 LEU2 ade2*
	PM27	*MATα*	*ura3 hom3* **his1-662** *arg6 trp2 leu2* ADE2
PM192	LZ10-8B	*MATa*	*ura3 HOM3* **his1-30** *ARG6 TRP2 LEU2 ade2*
	PM27	*MATα*	*ura3 hom3* **his1-662** *arg6 trp2 leu2* ADE2
PM193	HP067-4B	*MATa*	*URA3 HOM3* **his1-42** *ARG6 TRP2 LEU2 ade2*
	PM27	*MATα*	*ura3 hom3* **his1-662** *arg6 trp2 leu2* ADE2
PM195	HP007-3B	*MATa*	*URA3 HOM3* **his1-315** *ARG6 TRP2 leu2 ade2*
	PM27	*MATα*	*ura3 hom3* **his1-662** *arg6 trp2 leu2* ADE2
PM200	PM34	*MATa*	*ura3 HOM3* **his1-258,40** *ARG6 TRP2 LEU2 ade2*
	PM28	*MATα*	*ura3 hom3* **his1-672** *arg6 trp2 leu2* ADE2
PM201	PM35	*MATa*	*ura3 HOM3* **his1-258,30** *ARG6 trp2 LEU2 ade2*
	PM28	*MATα*	*ura3 hom3* **his1-672** *arg6 trp2 leu2* ADE2
PM300	HP077-2B	*MATa*	*ura3 HOM3* **his1-40** *ARG6 TRP2 LEU2 ade2*
	PM36	*MATα*	*ura3 hom3* **his1-258,672** *arg6 trp2 leu2* ADE2
PM301	LZ10-8B	*MATa*	*ura3 HOM3* **his1-30** *ARG6 TRP2 LEU2 ade2*
	PM36	*MATα*	*ura3 hom3* **his1-258,672** *arg6 trp2 leu2* ADE2

METHODS

4. *E. coli*

A. Growth

E. coli strain MV1190 was grown at 37°C either on LB or on 2x YT medium. Ampicillin was added to the media to a final concentration of 50-100 µg/ml whenever necessary. CJ236 was grown in the presence of chloramphenicol (30 µg/ml). Kanamycin was added to a final concentration of 70 µg/ml during isolation of single stranded DNA from the phagemids as suggested in the Mutagene *in vitro* mutagenesis kit.

B. Transformation

Competent *E. coli* cells were prepared by the standard procedure (Maniatis et al., 1982) and stored at -70° C after freezing with dry ice. 200 µl aliquots of cells were thawed and used for transformation.

5. Yeast:

A. Growth

All the yeast strains were grown at 30°C either on the plates or in liquid medium. The *his1-42* allele is temperature sensitive and strains heteroallelic for this markers are grown at 37°C for selection of prototrophic recombinants. For regular maintenance, *his1-42* was grown at 30°C. Sporulation was done at room temperature.

B. Genetic methods

The genetic procedures such as mating, isolation of diploids and auxotrophic marker identification were done following standard procedures (Sherman et al., 1982).

C. Random spore analysis

Diploids were grown in YPA liquid medium to near saturation and the cells were collected and washed twice with water. The diluted cells (about 10^8 cell/plate) were plated on sporulation medium plates.

When the sporulation was around 90% complete the sporulated cells were collected and suspended in 20% Glusulase solution. The suspension was digested for two hours. The digested spores were diluted in 5 ml water and passed through a French pressure apparatus twice. Separation of tetrad spores was monitored under a microscope. If the spores were still sticking together the suspension was agitated with sterile glass beads for one or two hours at 30°C to get individual spores.

The appropriately diluted spores were plated on solid minimal and complete media to assess the meiotic recombination frequency.

D. Unselected tetrad dissection

The sporulated cells were collected from the sporulation plates and suspended in a 10% Glusulase solution. After incubation for 10 minutes at 30°C the suspension was diluted with water and streaked onto YEPD or MC plates. The spores were dissected with a micromanipulator (Singer MSM micromanipulator). The plates were incubated for 2 to 3 days at 30°C and the prototrophs growing on MC-his plates were replica-plated onto MC-thr (to check for homoserine auxotrophy) and MC-arg plates to determine crossovers and non-crossovers among the prototrophs.

E. Allele testing

The identity of *his1* alleles in the dissected spores was determined by crossing clones of each spore to appropriate tester strains and checking for histidine prototrophy resulting from mitotic recombination. There should be no prototrophs produced if the spores contained the same allele as the tester. Thus, the prototrophs

produced, if any, would be low in frequency and would be equivalent to the reversion frequency. Both haploid parents and the diploid used in the original cross were used as controls.

F. Transformation

Yeast transformation was done according to Hinnen et al. (1978) with slight modifications using lithium acetate. A well isolated colony was grown in 5 ml YEPD over night and 200 µl of the overnight culture was diluted in 50 ml YEPD and grown to 10^7 cells per ml. The cells were harvested, washed once with water, and resuspended in 1 ml 0.1M Tris-HCl, 0.01 M EDTA, and 0.1 M LiAc pH 7.5 to a concentration of 2 X 10^9 cells per ml. For each transformation, 50 µl of cell suspension (~10^8cells) was added to the solution containing transforming DNA (amount varies from 0.5 to 5 µg) and carrier DNA (5 µg) in a microfuge tube. To this mixture, 0.6 ml of PEG solution (40% PEG 4000, 0.1M LiAc in TE) was added and the mixture was incubated at 30°C for 30 minutes. After heat shock at 42°C for 15 minutes the cells were washed twice with TE at pH 7.5 and incubated in YEPD for 3 hours. The cells were harvested, washed with water twice, and plated on appropriate plates. The plates were incubated for 3-4 days at 30°C to get transformants. These transformants were checked by streaking on the appropriate omission media and then restreaked on YEPD to get individual clones. The individual clones were checked again for their markers and these clones were used for further analysis.

6. DNA Manipulations

A. Plasmid isolation and restriction

Preparation and storage of plasmid DNA, restriction analysis, sub-cloning and agarose-gel electrophoresis were performed according to standard methodology (Maniatis et al., 1982).

B. Single -stranded DNA isolation from phagemids

The single-stranded DNA for *in vitro* mutagenesis was isolated from a 2.5 ml minipreparation instead of large scale preparation as mentioned in the Mutagene protocol kit.

The plasmid pHP1 was transformed into CJ236 competent cells. A well isolated transformed colony of CJ236 on an ampicillin plate was inoculated in 2X YT medium containing 50 µg/ml ampicillin and 30 µg/ml chloramphenicol. This was grown at 37°C for one hour and the helper phage (M13KO7) was added to a final concentration of 10^7 plaque forming units per ml (pfu) and continued incubation for another hour. Kanamycin was added to a final concentration of 70 µg/ml and incubated overnight. The cells were pelleted in an eppendorf tube for 5 minutes. To the supernatant, PEG2000 and 7.5 M ammonium acetate were added to a final concentration of 4% and 0.8 M respectively and left on ice for one hour. This was centrifuged at 4°C for 10 minutes. The supernatant was drained and the pellet with single-stranded DNA was suspended in 200 µl of high salt buffer (300 mM NaCl, 100mM Tris, pH 8.0, 1mM EDTA). The DNA was extracted with phenol/chloroform (1:1) mixture 4 times and twice with chloroform/isoamyl alcohol (24:1). To the cleaned supernatant 1/10 volume of ammonium acetate and 2.5 volume of ethanol were added and kept at -70°C for 45 minutes to one hour. This was centrifuged again at 4°C for 20 minutes and the pellet was washed once with 70% ethanol and vacuum dried. The dried pellet was dissolved in distilled water and the amount of DNA was measured with spectrophotometer. For each *in vitro* mutation reaction approximately 0.5 µg of DNA was used.

C. Site-specific oligonucletide-directed *in vitro* mutagenesis

The steps involved in *in vitro* mutagenesis are described in FIG. 4. The phagemid with insert (pHP1) was transformed into *E. coli* strain CJ236. Single-stranded DNA was isolated after super infecting with helper phage M13 K07. As CJ236

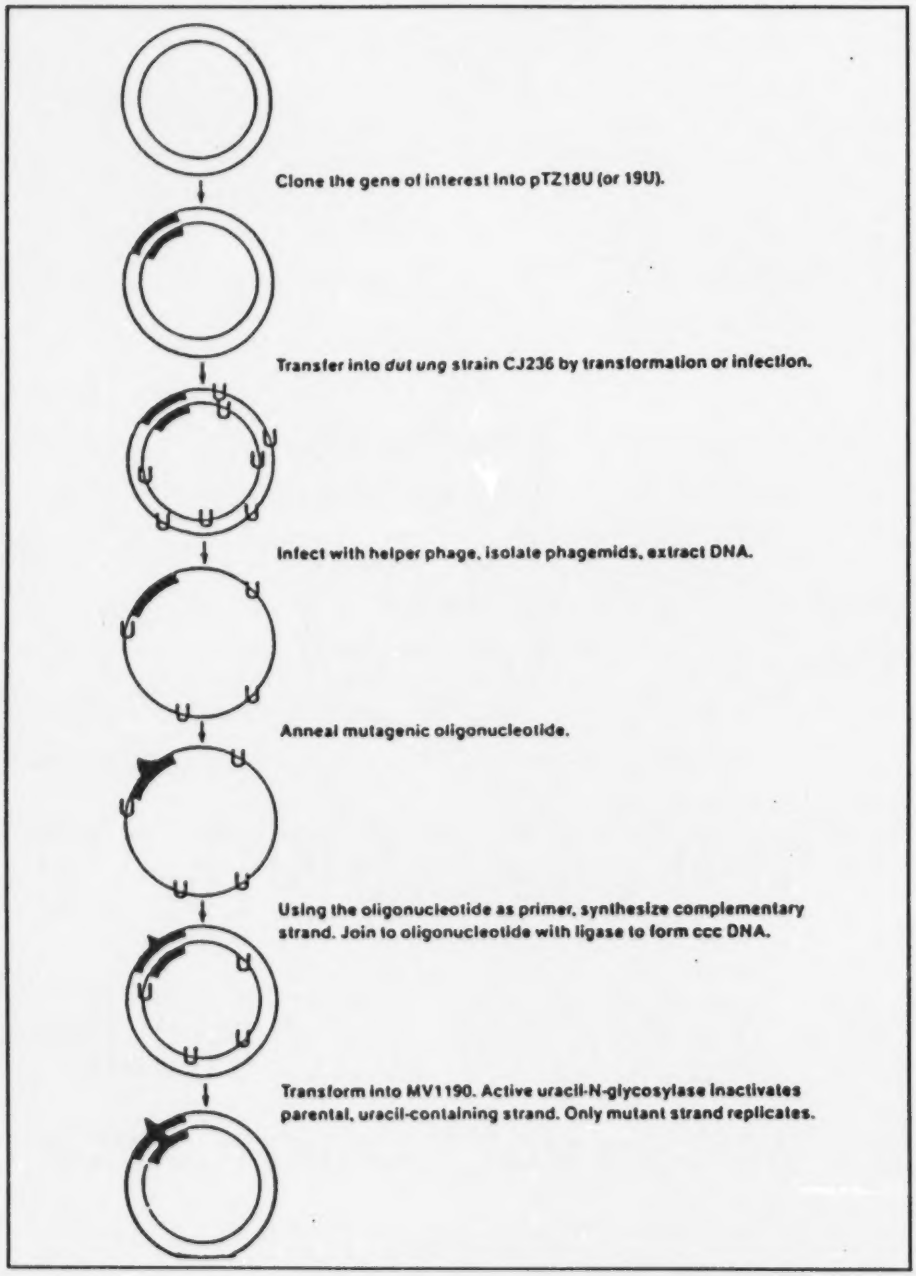
Clone the gene of interest into pTZ18U (or 19U).
Transfer into *dut ung* strain CJ236 by transformation or infection.
Infect with helper phage, isolate phagemids, extract DNA.
Anneal mutagenic oligonucleotide.
Using the oligonucleotide as primer, synthesize complementary strand. Join to oligonucleotide with ligase to form ccc DNA.
Transform into MV1190. Active uracil-N-glycosylase inactivates parental, uracil-containing strand. Only mutant strand replicates.

FIG. 4. Steps involved in *in vitro* mutagenesis using phagemid vectors (Bio-Rad Lab instruction manual).

was deficient in dUTPase (*dut*-) and uracil N-glycosylase (*ung*-) the single-stranded DNA produced from this strain would carry a number of uracils (U-DNA). This DNA is inactivated in the host with an active glycosylase (MV1190). As this DNA was used as a template for oligonucleotide-primed *in vitro* DNA synthesis of the complementary strand, after transformation into MV1190, the template strand was preferentially eliminated leaving the newly *in vitro* synthesized strand. The primer with the mutation would create a mutation as shown in FIG. 4.

D. Yeast DNA isolations

Yeast DNA was prepared for Southern analysis and PCR amplification as follows: a separate colony was suspended and grown in 5 ml YEPD overnight. The cells were spun down and washed once with water and suspended in 0.5 µl 1M sorbitol and 0.1M EDTA with 20 µl of 2 mg/ml Zymolyase (20T). The suspension was incubated at 37°C for an hour. The digested cells were suspended in 500 ml 20mM Tris and 50mM EDTA. An equal volume of PCI (phenol:chloroform:isoamylalcohol 25:24:1) was added and vortexed with maximum speed for a minute and centrifuged for 4 minutes. The supernatant was washed once with chloroform:isoamyl alcohol (24:1). The DNA was precipitated in an equal volume of isoamyl alcohol and dissolved in 50 µl T.E.

E. Southern blotting

All gels for Southern analysis were blotted onto Gene Screen Plus membrane using the capillary blot protocol recommended by the manufacturer (Du Pont Co.). All radio-isotopically labelled materials were purchased from New England Nuclear laboratories.

F. PCR amplification

The genomic DNA isolated from small scale preparations as mentioned above was used for PCR amplification. For amplification the master mix (90%) was prepared

as follows, stored at -20°C and used whenever necessary (Gary Ritzel's personal communication)

90%PCR mix:

Tris pH9.0	1M	= 50 µl
$MgCl_2$	1M	= 6 µl
Am. sulphate	1M	= 15 µl
2Mercapto-ethanol	14.5M	= 1 µl
dNTPs	100mM	= 5 µl each
Primers	1 µg/µl	= 5 µl each
BSA 20 mg.ml		= 5 µl
H2O to		1 ml.

The reaction mix consisted of 27 µl of 90% PCR mix and 3 µl of a solution containing 0.2 to 3 µg of template DNA and 1 unit of *Taq* polymerase. A drop of paraffin oil overlay was added to prevent evaporation.

The amplification cycles were as follows:

The initial denaturation was for 4 minutes at 95°C and the following cycles were for 4 minutes at 93°C. The amplification cycles consisted of 10 seconds denaturation, 30 sec annealing at 58°C and 1 minute extension at 72°C. After 30 cycles of amplification the amplified DNA was run out in 1% agarose gel and isolated by Gene Clean for further analysis.

C. PCR sequencing

About 1 to 2 µg of PCR amplified double-stranded DNA template and 100 ng of primers were boiled in 13 µl water for 5 minutes and frozen immediately in dry ice.

The master mix was prepared from the Sequenase kit (supplied by United States Biochemicals) as follows: Mn buffer (0.15M Sodium Isocitrate,0.1M $MnCl_2$) 1 µl, DTT

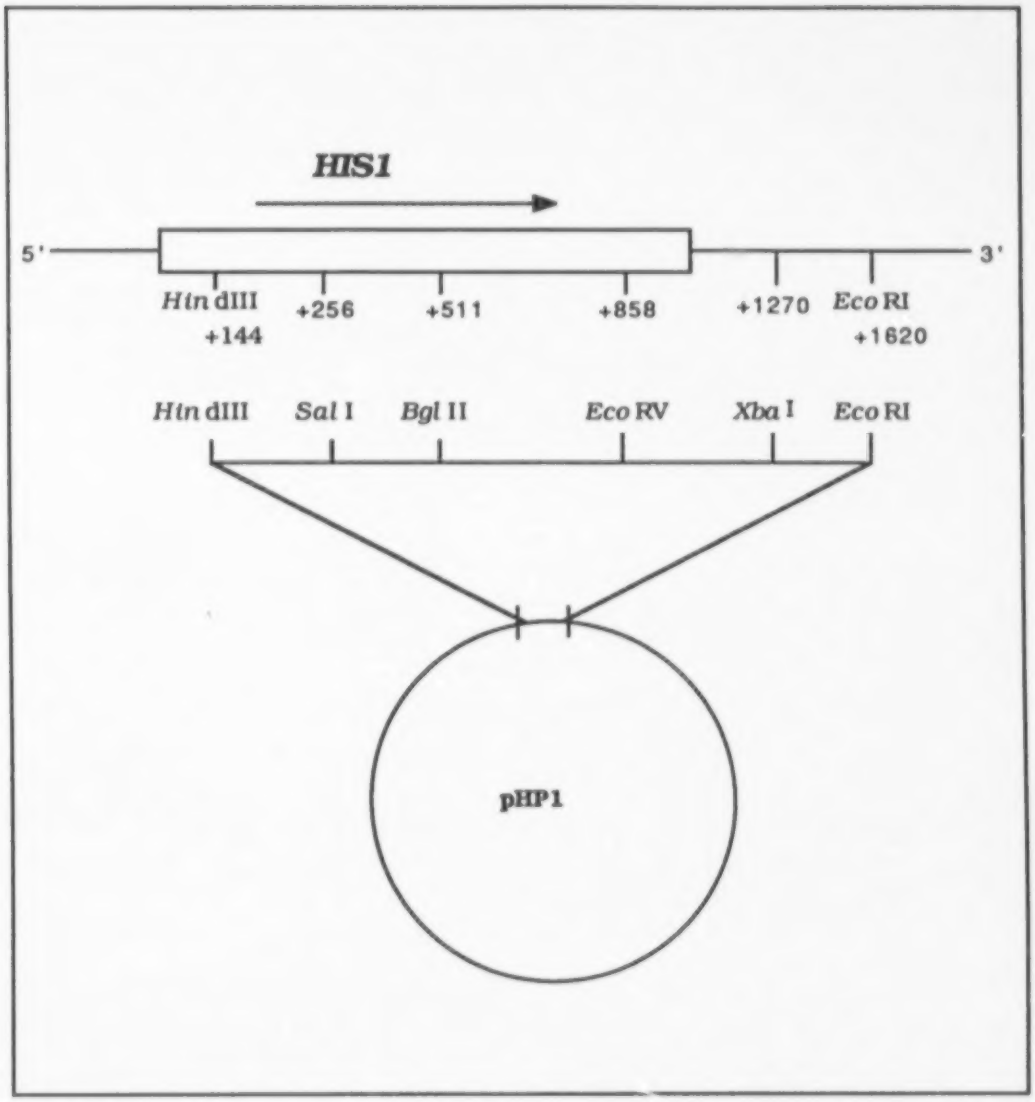

FIG. 5. Subcloning of the *HIS1* fragment in the phagemid (PTZ18) for *in vitro* mutagenesis.

The unique restriction sites on the plasmid, shown above, represent the sites on the cloned fragment.

(0.1M) 1 µl,1:2 diluted labelling mix (5X labelling mix consisted of 7.5mM each of dCTP, dGTP and dTTP) 2 µl , [α-^{35}S] dATP 0.5 µl and 2.5 units of Sequenase enzyme.

Frozen template with primer mix was thawed at room temperature for a minute and centrifuged very briefly. 4.5 µl of master mix was added to the primer mix template, centrifuged very briefly and kept for 2 minutes at room temperature for annealing. The reaction mix was divided into 4 Eppendorf tubes labelled as A, C, G and T and 2.5 µl di-deoxy mix, which was supplied with the kit, was added and left at 37°C for 5 minutes. Four µl of stop solution (95% formamide, 20mM EDTA, 0.05% bromophenol blue and 0.05% xylene cynol) was added finally to stop the reaction. The reaction mix was boiled for 5 minutes leaving the cap opened before loading on to the sequencing gel. The sequencing was carried out following Maniatis et al. (1982).

7. *In vitro* construction of *his1* mutants

A. *HIS1* subcloning

All the *in vitro* constructs were made in the phagemid containing the fragment of the *HIS1* gene. The strategy for cloning the *HIS1* gene fragment is shown in FIG. 5. A 1.5 kb fragment (*Hin*dIII-*Eco*RI) spanning from position +144 to the stop codon (+897) with 723 bp of 3' sequence of the *HIS1* gene from the plasmid pHIS1 was cloned in the phagemid PTZ18 (2.8 kb). By cloning, the multi-cloning site in the PTZ18 plasmid was eliminated. The resultant plasmid pHP1 has *Eco*RV and *Bgl*II sites which are unique to the *HIS1* fragment. As the *Sal*I site on PTZ18 was eliminated during cloning, the new unique *Sal*I site on the plasmid pHP1 is also from the *HIS1* insert (FIG. 5 and Appendix 1). The plasmid pHP1 (4.3 kb) was used for further manipulations. All the steps, including cloning, *in vitro* mutagenesis, *in vivo* disruption, *in vivo* placement of *his1* alleles on the yeast chromosome and confirmation of gene replacement by PCR amplification and DNA sequencing for the allele *his1-876* are shown in the appendices

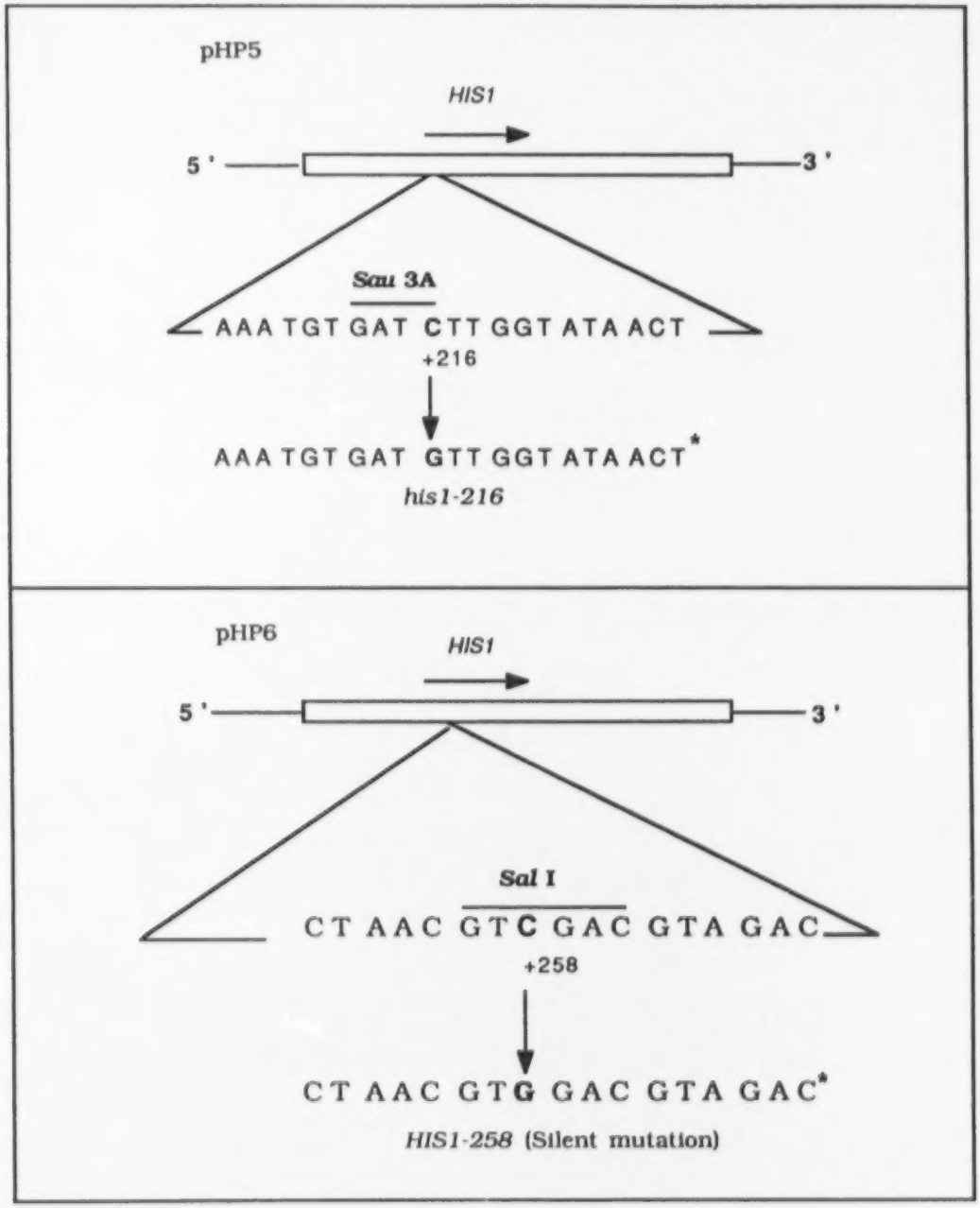

FIG. 6. Construction of *his1-216* (pHP2) and *HIS1-258* (pHP6) mutations in a cloned *HIS1* gene by *in vitro* mutagenesis.

* Primer used for *in vitro* mutagenesis.

1 to 5. The steps are similar for the other newly created *his1* alleles also and the strategy for construction of these alleles is described below.

Three types of *in vitro* manipulations were made: (i) base substitutions by oligonucleotide directed mutagenesis, (ii) elimination of restriction sites by filling-in, and (iii) *in vitro* disruption of *HIS1* with *URA3* at *Sal*I and *Eco*RV sites. All the manipulations were done with the plasmid pHP1.

B. Base substitutions by oligonucleotide directed mutagenesis

The strategy for *in vitro* mutagenesis is shown in FIG. 4. This was done by making single-strand DNA from the plasmid pHP1 by transforming *E. coli* CJ236 and super-infecting with helper phage (M13 K07). Artificially synthesized oligonucleotides incorporating the relevant mutations were used as primers for *in vitro* synthesis of the second DNA strand. After mutagenesis and transformation, three randomly selected colonies were usually characterized for the presence of mutation as revealed by gel electrophoresis. Typically, two of them showed the presence of the mutation. These mutations were confirmed finally by sequencing after replacement on the yeast chromosome. The following mutations were made by *in vitro* mutagenesis.

pHP5 (*his1-216*)

This is a C to G transversion mutation at +216. A 21 base oligonucleotide was used as a primer for *in vitro* mutagenesis. The mutation abolishes the *Sau*3A site (FIG. 6). The presence of the mutation was confirmed in two steps: i) restriction of the plasmid with *Hin*dIII and *Sal*I to liberate a 0.36 kb fragment and ii) restriction of the 0.36 kb fragment with *Sau*3A. As there is one *Sau*3A site present in that interval, two bands were seen in the wild type and only one band (uncut) was seen in the mutant.

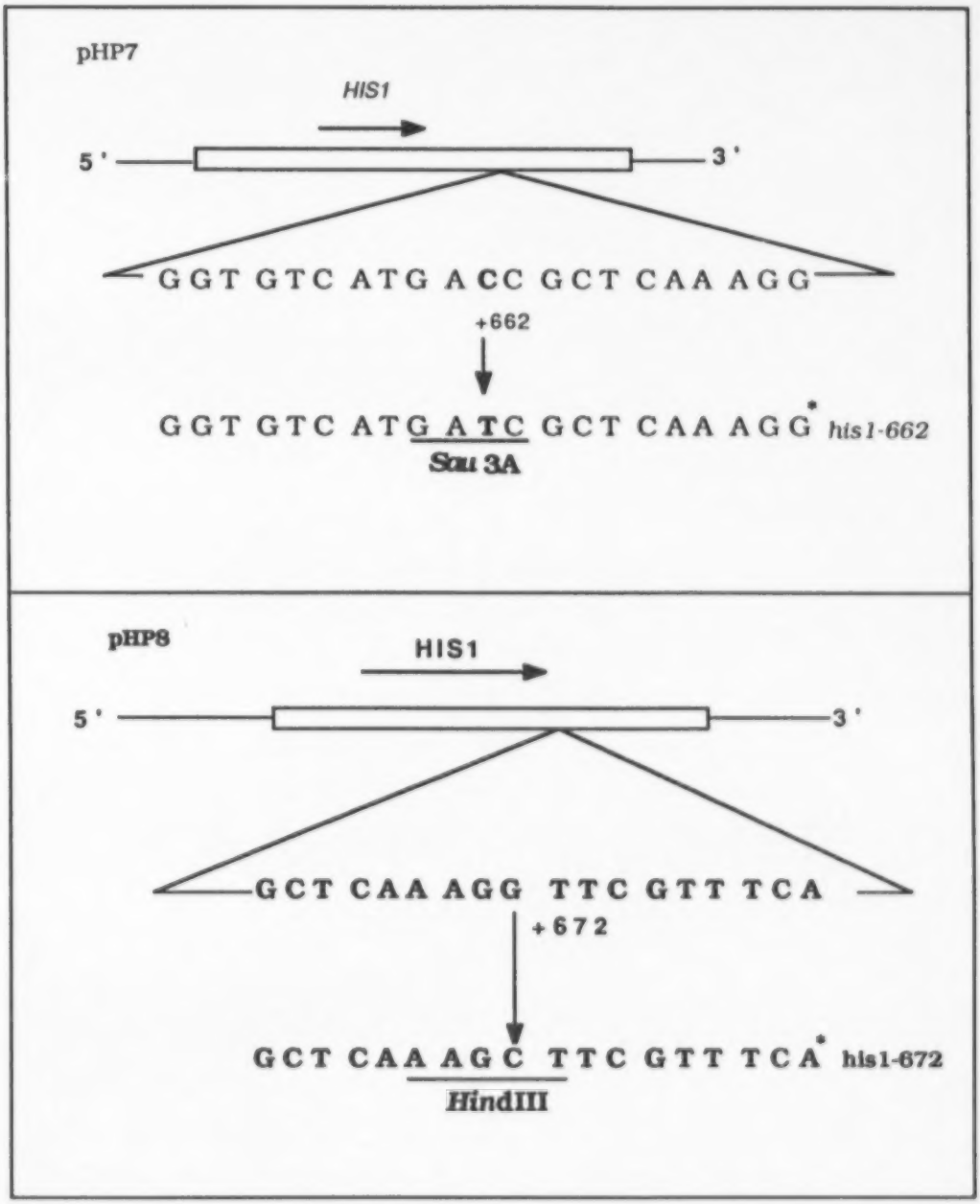

FIG. 7. Construction of *his1-662* (pHP7) and *his1-672* (pHP8) mutations in a cloned *HIS1* gene by *in vitro* mutagenesis.

* Primer used for *in vitro* mutagenesis.

The mutagenesis protocol is shown in FIG. 6. For *in vitro* mutagenesis, a 17 base oligonucleotide was used as a primer. This is a silent mutation and eliminates a unique *Sal*I site on the pHP1 plasmid. As pHP1 has only one *Sal*I site the mutation was confirmed by the absence of a cut by *Sal*I.

pHP7 (*his1-662*).

This is a transversion mutation from G to T at +662. The primers used and the strategy for *in vitro* mutagenesis are shown in FIG. 7. This mutation was confirmed in two steps by identifying a new *Sau*3A site. First, the plasmid was cut by *Bgl*II and *Eco*RV enzymes to liberate a 0.35 kb fragment. In the next step, the isolated fragment was cut by *Sau*3A. The mutation showed two bands while the wild-type showed only one uncut band.

pHP8 (*his1-672*)

This is a G to C transversion mutation at position +672. For mutagenesis, an 18 base oligonucleotide was used as a primer. This mutation creates a new *Hin*dIII site on the pHP1 plasmid (FIG. 7). Restriction with *Hin*dIII liberated a 0.52 kb fragment if the mutation was present. There was a single cut on the plasmid without the new mutation.

pHP9 (*his1-876*)

This mutation is a palindromic insertion of 28 bases (14 bp direct repeat). The oligonucleotide used for mutagenesis is shown in FIG. 8. The palindromic insertion created an *Apa*I site at position +876. This is a unique site and digestion with *Apa*I linearized the mutated plasmid pHP9 (Appendix 2).

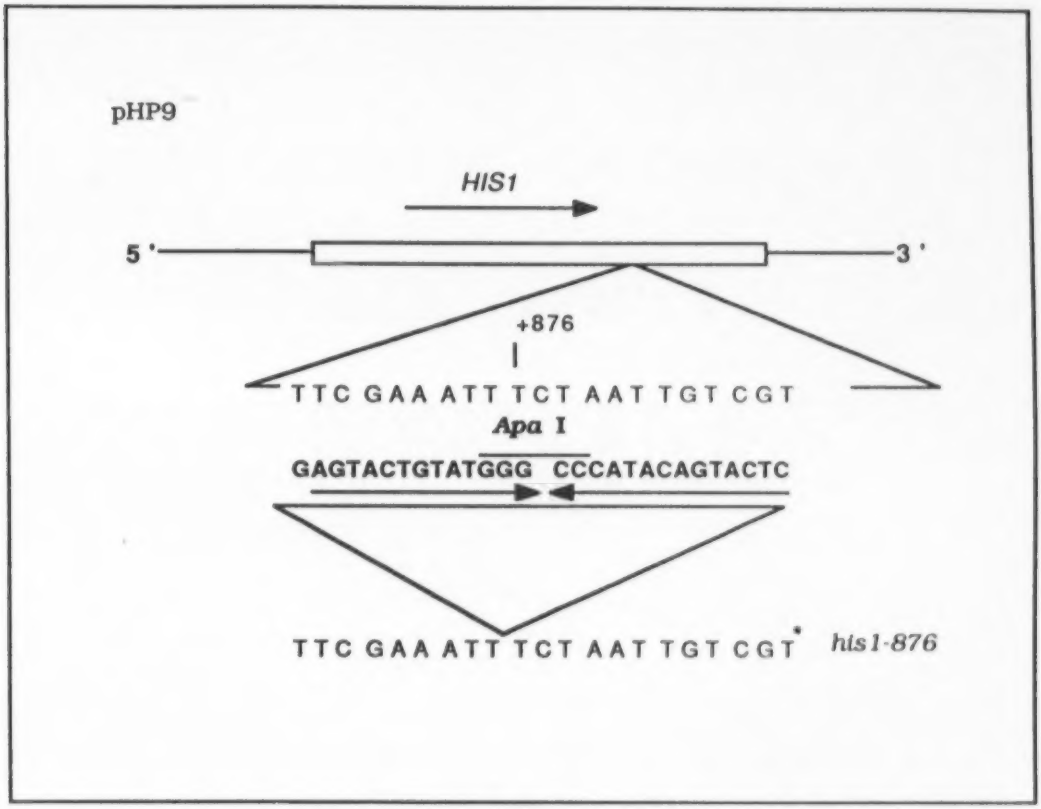

FIG. 8. Construction of the *his1-876* mutation (pHP9) in a cloned *HIS1* gene by *in vitro* mutagenesis.

* Primer used for *in vitro* mutagenesis.

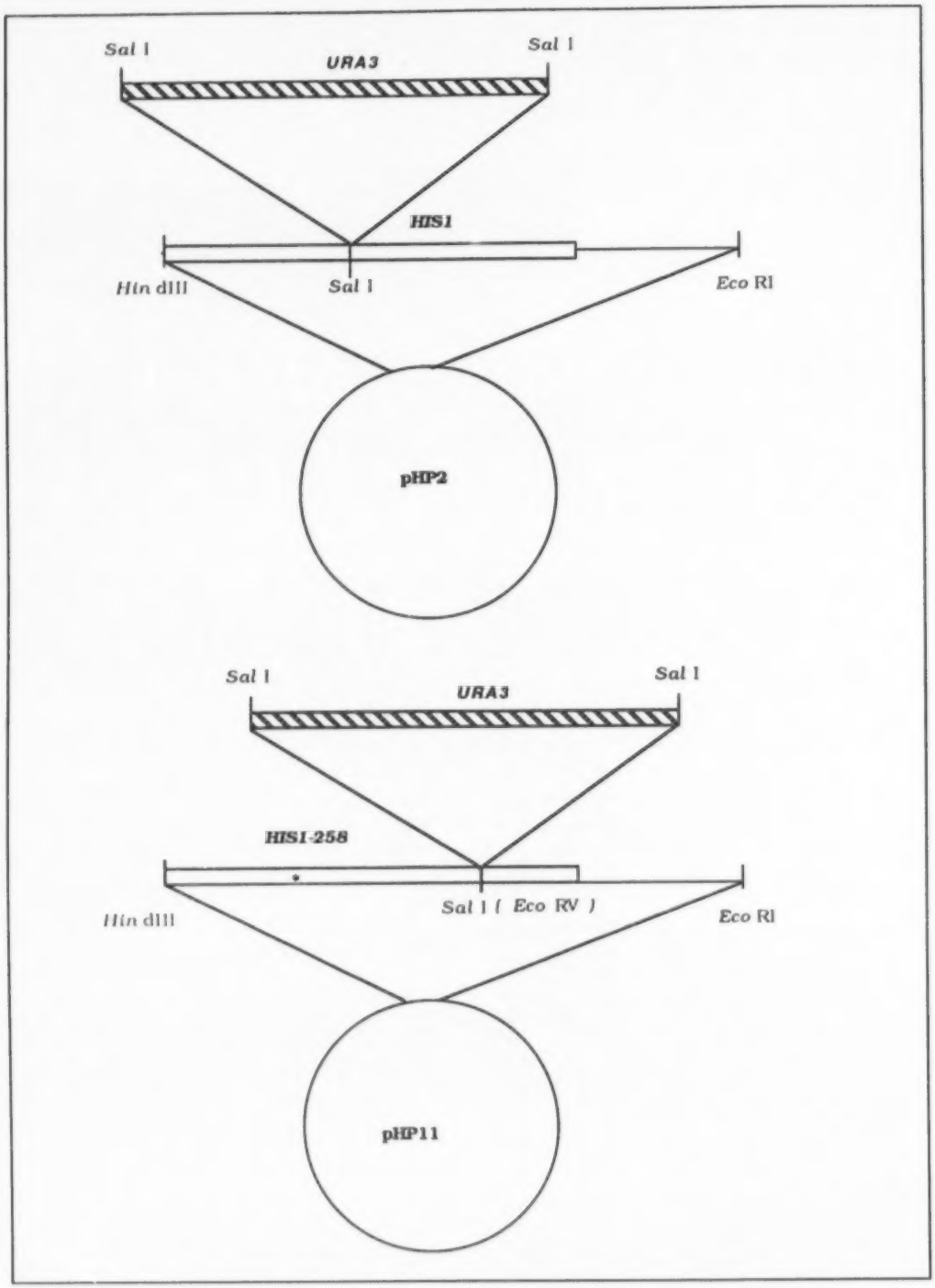

FIG. 9. *In vitro* disruption of a cloned *his1* gene at the *Sal*I (pHP2) and *Eco*RV sites (pHP11).

C. Elimination of restriction site mutations

The plasmid pHP1 was cut by *Sal*I or by *Bgl*II and the sticky end was filled-in and ligated by standard procedures. The *Sal*I site is present at +260 and *Bgl*II at +511. This filling-in procedure destroyed the *Sal*I (pHP3) and *Bgl*II (pHP4) sites. Both of them were unique sites on the pHP1 plasmid (FIG. 5) and the absence of sites confirmed the filling-in.

D. *In vitro* disruption of *HIS1* with *URA3*

Disruption at the *Sal*I site

The strategy for *in vitro* disruption is shown in FIG. 9. For this, the *URA3* gene from plasmid YCp50 was used and the disruption was done in two steps. Plasmid YCp50 was cut with *Sma*I which opened up the plasmid leaving blunt ends. A *Sal*I linker was ligated to the blunt ends. A second digestion with *Sal*I liberated a 1.9 kb *URA3* fragment (from the *Sma*I to the *Sal*I sites on YCp50) with sticky ends. In the next step pHP1 was restricted with *Sal*I to linearize the plasmid. The 1.9 kb fragment with the *URA3* gene was ligated to this linearized pHP1 plasmid to result in a 6.29 kb plasmid (pHP2). Restriction with *Sal*I liberated the *URA3* fragment showing bands of 4.3 and 1.9 kb. There are unique *Apa*I and *Nco*I sites on the *URA3* fragment which showed a single band on the gel after digestion with those enzymes.

Disruption at the *Eco*RV site

This was also done in two steps. A *Sal*I linker was inserted into the *Eco*RV site of pHP6 to get pHP10. There was no *Sal*I site in pHP6 and the new *Sal*I site in pHP10 is at the *Eco*RV site of pHP6. Insertion of this linker eliminated the *Eco*RV site. The *URA3* fragment, cut from pHP2 by *Sal*I, was inserted into the newly created *Sal*I site in pHP10 to get pHP11 (FIG. 9).

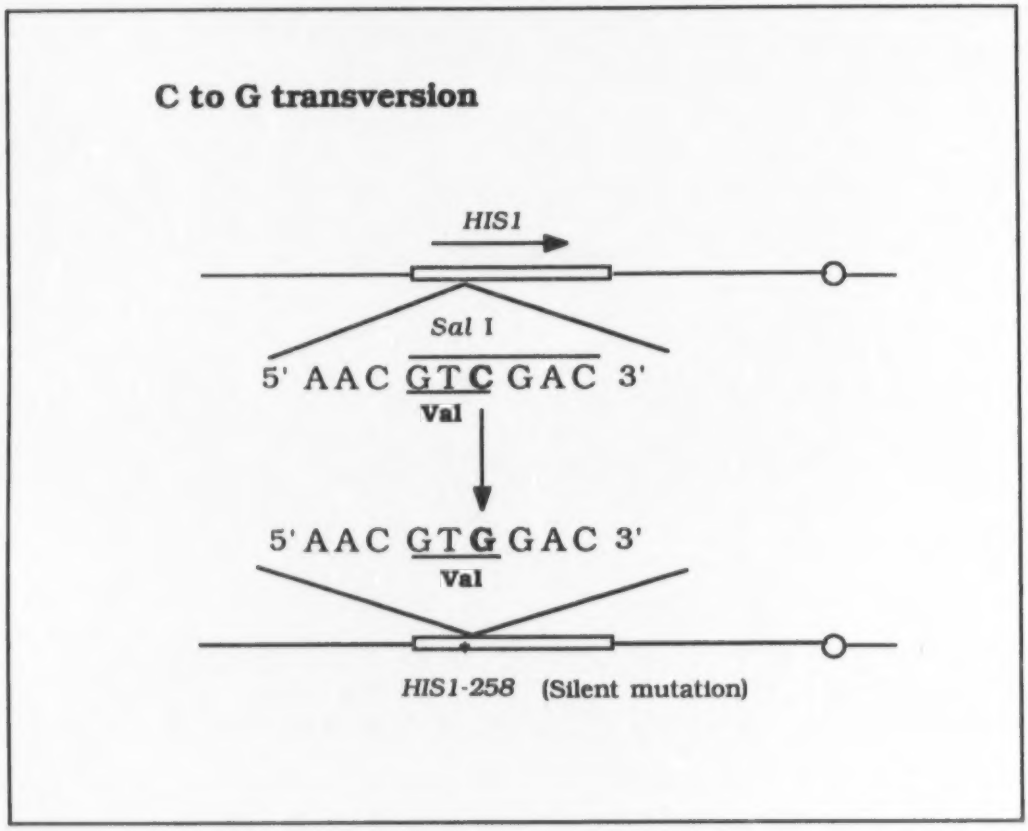

FIG. 10. Construction of the *HIS1-258* allele on the yeast chromosome by gene replacement.

8. *In vivo* placement of *his1* mutants into yeast

All the *his1* mutants, except *HIS1-258*, were incorporated *in vivo* by a two step replacement. The first step was disruption of *HIS1* and the next was replacement of the disrupted gene with a fragment having a mutation obtained from the *in vitro* mutagenized plasmids. *HIS1-258* was inserted using only one step. This replacement was done with a haploid having the *his1-49* deletion mutation (PM1-19). The *Hin*dIII to *Bgl*II fragment from plasmid pHP6 was used for transformation. The transformants were selected on MC-his plates since the phenotype of this mutation is His^+. The change was made at the third position of the codon (GTC to GTG) so that no change is expected in the amino acid encoded (Valine). The C to G transversion abolished the *Sal*I site (FIG. 10). The mutation was first confirmed by a polymerase chain reaction (PCR) amplification (primers for amplification are shown in Appendix 4) of the genomic DNA and restriction of the amplified DNA with *Sal*I and later by sequencing. A primer spanning from +206 to +227, which was used for *in vitro* mutagenesis to create the *his1-216* mutation (FIG. 6), was used for sequencing. The transformants from the MC-his plates were selected and restreaked to get single clones and these clones were amplified twice for restriction and a third time for sequencing. Though the fidelity of PCR amplification by *Taq* polymerase is fairly low, testing two or three times ruled out any PCR artifacts by *Taq* polymerase.

A. *In vivo* disruption of *HIS1*

Disruptions at different sites (*Sal*I or *Eco*RV site) were made for construction of either single or double mutants. For disruption, the *HIS1* fragment with the *URA3* gene in the middle is used for transformation and disruption was done by ends-out integration of *URA3*. The transformants were selected on MC-ura plates. The presence of *URA3* in the right place was confirmed by Southern analysis (Appendix 3).

Disruption at the *Sal*I site

A 2.4 kb fragment having the *URA3* gene at the *Sal*I site flanked by *HIS1* sequences (144 bp on the 5' side from the *Hin*dIII to the *Sal*I sites and 250 bp from the *Sal*I site to the *Bgl*II site on the 3' side) was used for disruption. Disruption was made with three different haploids for different purposes which are explained later. The haploid strain PM21 resulted from disruption of wild-type strain (LZ21-13C). Two other mutant strains, *his1-40* (HP077-2B) and *his1-30* (LZ10-2A), were also used for disruption to get PM32 and PM33, respectively. The transformants were selected on MC-ura plates. The phenotype of the disrupted strains is Ura^+ and His^-. This strategy was followed for the *his1-30* (LZ10-2A) disruption also since *his1-30* is a temperature-sensitive allele and can be grown at room temperature without histidine supplementation. For *his1-40* (HP077-2B) this could not be followed since the phenotype of this mutant is His^-. In the disrupted strain PM32 (*his1-40*) and PM33 (*his1-30*), the mutations *his1-40* and *his1-30* were not disturbed since the fragment used for disruption excludes these alleles. The disruptions were confirmed by southern blot analysis. The Southern blot analyses showed that *Eco*RI enzyme digestion gave a fragment of about 8 kb in the control and the insertion of *URA3* gave a fragment of approximately 10 kb.

Disruption at the *Eco*RV site

Two strains were used for disruption at the *Eco*RV site. One was wild-type at *HIS1* (LZ21-13C) and the other one was *HIS1*-258 (PM26). As both these strains were His+, the presence of *URA3* was identified from the Ura^+His^- phenotype. For transformation, the *Bgl*II to *Xba*I fragment of the *HIS1* gene with the *URA3* gene at the *Eco*RV site from the plasmid pHP11 was used. These disruptions were also confirmed by Southern blot analysis.

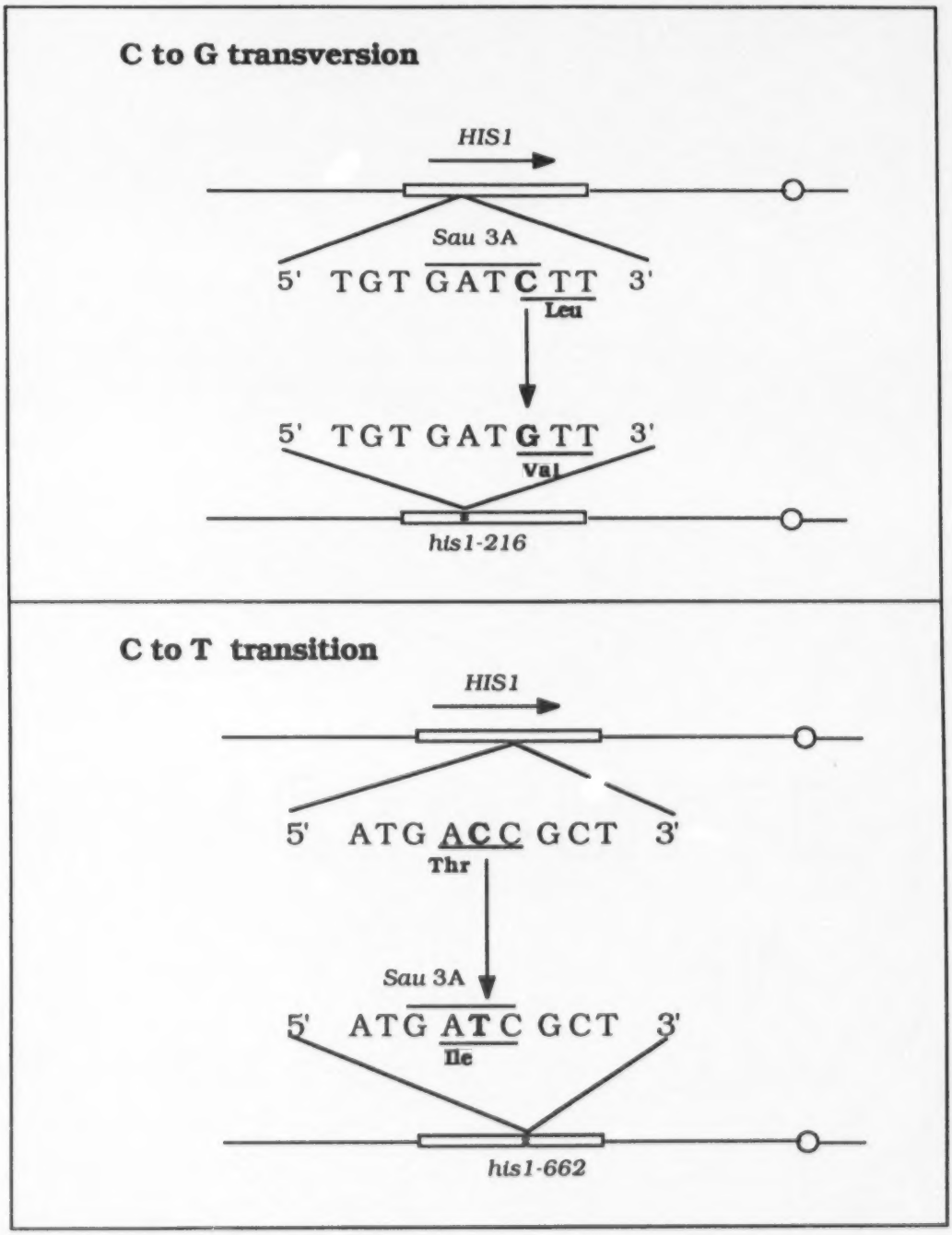

FIG. 11. Construction of *his1-216* and *his1-662* alleles on the yeast chromosome by gene replacement.

B. Replacement of *HIS1* alleles

For replacement, the disrupted strains were transformed with the DNA fragment containing the mutation. The plasmid with the mutation was cut by appropriate enzymes and the fragment containing the mutation was isolated from agarose gel and used for transformation. Typically 2-3 μg of DNA were used for each transformation. The replacement eliminated the *URA3* gene from the *HIS1* locus hence the transformants were selected on FOA plates which allows only Ura$^-$ clones to grow. On each FOA plate 50-100 colonies were obtained per transformation. Those colonies were re-streaked on YEPD plates to get single clones. Typically, six colonies isolated from different transformants were tested for the presence of the mutation. As all the mutations have restriction sites as markers, the mutations were identified by PCR amplification of the genomic DNA and restriction with appropriate enzymes. This was confirmed by another round of PCR amplification and restriction to avoid PCR artifacts. Opposite primers spanning from +1 to +22 (PHA15) and from +1069 to +1080 (PHA14) were used (Appendix 4) for PCR amplification. The confirmed colonies were used for further analysis.

The following strains were constructed by gene replacement with PM21 (disruption of *HIS1* at *Sal*I site):

PM25 (*his1-216*)

This is a C to G transversion mutation at +216. The mutation abolishes the *Sau*3A site. The *Hin*dIII-*Bgl*II fragment containing this mutation was isolated from the plasmid pHP5 and used for transformation. The transformants were verified by a two step analysis. In the first step the PCR amplified DNA was cut with *Hin*dIII and *Bgl*II to liberate the 0.37 kb fragment. In the next step the isolated fragment was restricted with *Sau*3A. In the control, two bands were seen while in the strain with the mutation only

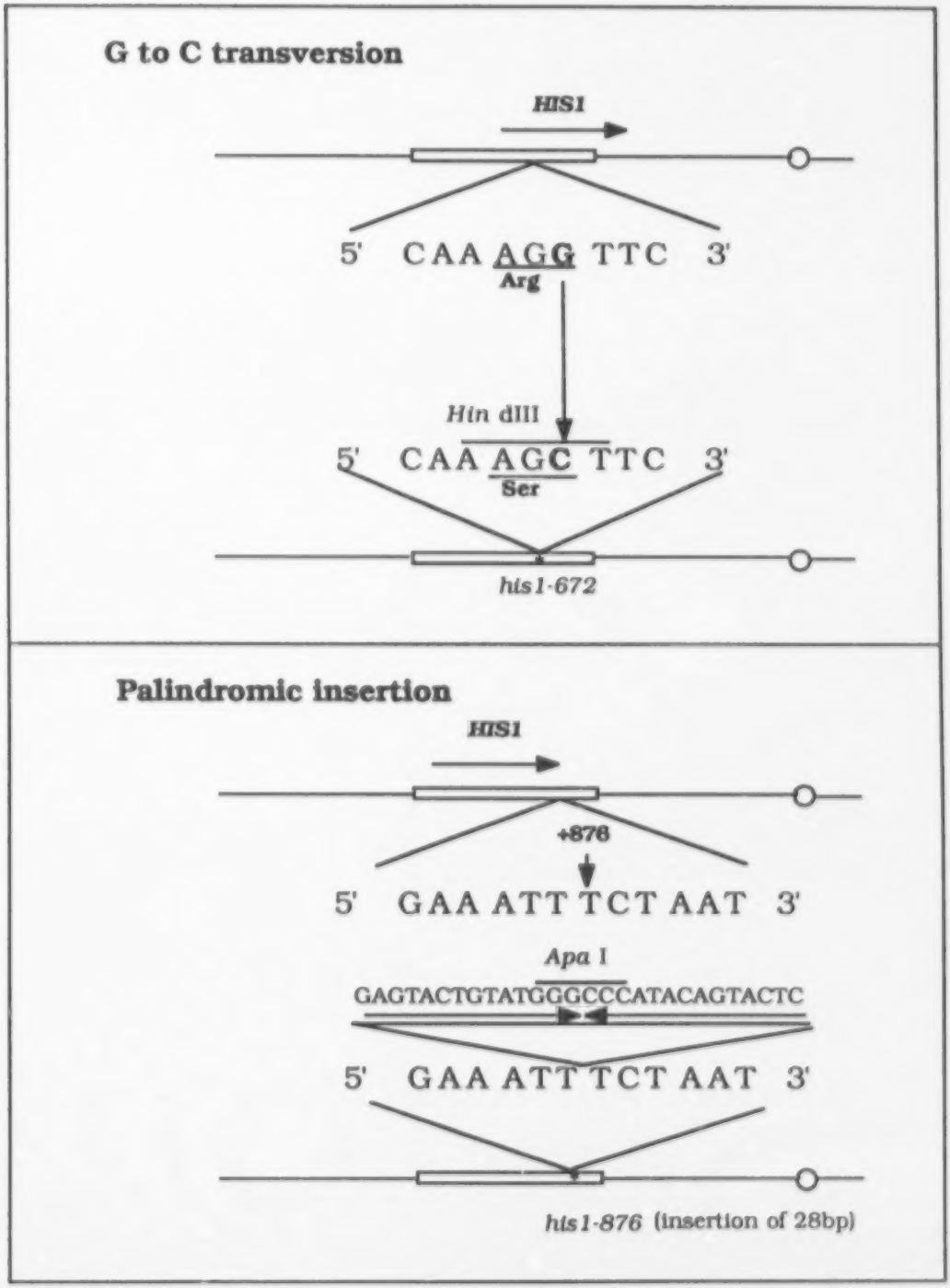

FIG. 12. Construction of *his1-672* and *his1-876* alleles on the yeast chromosomes by gene replacement.

one band was seen. This mutation is expected to change the amino acid from leucine to valine (FIG. 11) and the phenotype is His$^-$.

PM27 (*his1-662*)

This is a G to T transversion mutation. The plasmid pHP7 was cut by *Hin*dIII and *Xba*I to liberate a 1.13 kb fragment. This fragment was used for replacement. This too was selected on FOA plates. This mutation creates a new *Sau*3A site (FIG.11) and the mutation was confirmed by PCR amplification of the genomic DNA and sequencing with a primer spanning from +694 to +710. The amino acid change is expected to be from threonine to isoleucine. The phenotype of this mutant is His$^-$.

The following single-site mutations were constructed by replacement using strain PM22 (*HIS1* disrupted at the *Eco*RV site):

PM28 (*his1-672*)

The plasmid pHP8 was cut by *Bgl*II and *Xba*I to liberate a 0.759 kb fragment. The isolated fragment was used for transformation. This is a C to G transversion mutation and creates a new *Hin*dIII site (FIG. 12). The mutation was confirmed by PCR amplification and sequencing with a primer spanning from +694 to +710. The mutagenesis is expected to change the amino acid from arginine to serine. The phenotype of this mutant is His$^-$.

PM29 (*his1-876*)

This is a palindromic insertion mutation at the 3' end of *his1*. The insertion of the 28 bp palindrome (14 bp repeat) creates a new *Apa*I site (FIG.12). The *Bgl*II-*Xba*I fragment from pHP9 was used for replacement. This was confirmed by PCR amplification of the genomic DNA and sequencing (Appendices 4 and 5). As there is a large insertion, the phenotype of this mutant is His$^-$.

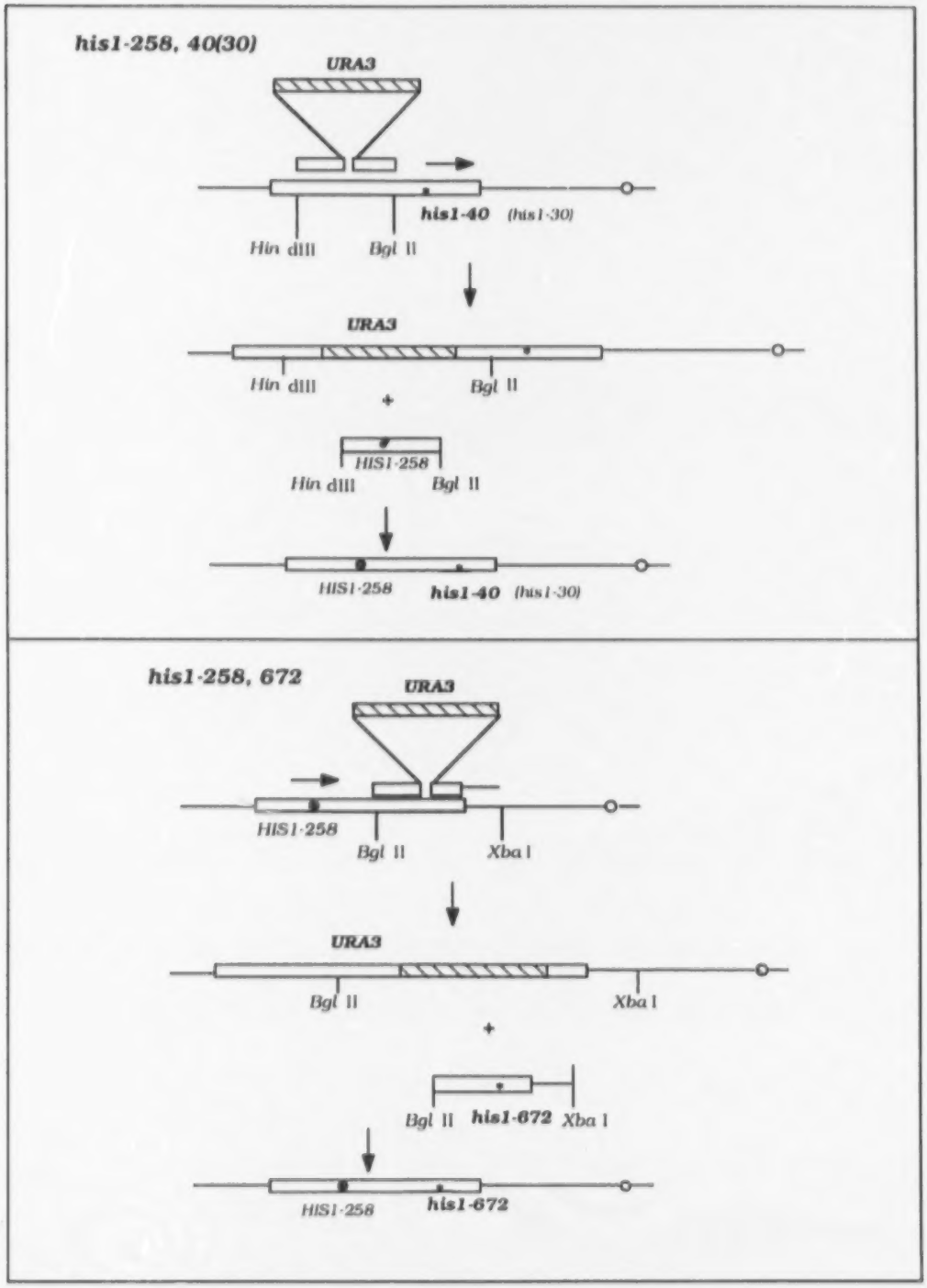

FIG. 13. Construction of double mutants on the yeast chromosome by gene replacement.

9. Construction of double mutants

The purpose of making double mutants is explained later. For making double mutants, the disruptions were made in the strains which already have a single mutation. The fragments used either for disruption or for replacement excluded those sites so that the existing mutations were not disturbed. All the double mutants carry *HIS1-258* as one of the alleles and *his1-40* (or *his1-30*) as the other allele.

PM34 (*his1-258,40*)

To construct this double mutant (*his1-258,40*), the haploid strain PM32 was used for replacement. PM32 has the *URA3* disruption at the *Sal*I (+260) site and the *his1-40* mutation at +683. The fragment from *Hin*dIII to *Bgl*II with the *HIS1-258* mutation in the middle, was used for transformation (FIG. 13). After replacement, the resultant haploid strain will have two mutations *HIS1-258* and *his1-40*. The transformants were analyzed by PCR amplification of the genomic DNA and the mutation was confirmed by restriction with *Sal*I.

PM35 (*his1-258,30*)

The haploid strain PM33 was used to construct this double mutant. As *his1-30* is located at +652, the fragment used for replacement from *Hin*dIII (+144) to *Bgl*II (+511) did not include this allele (FIG. 13). The replacement with *HIS1-258* makes PM33 a double mutant. Though *HIS1-258* is His^+, *his1-30* makes this double mutant His^-. The presence of *HIS1-258* was confirmed by the elimination of the *Sal*I site.

HPY36 (*his1-258,672*)

To construct the double mutant strain, PM31 (which has the *HIS1-258* mutation and a disruption at the *Eco*RV site) was used. The *Bgl*II to *Xba*I fragment from the pHP8 plasmid (which has a G to C transversion mutation at 672) was used for transformation

(FIG.13). The replacement was confirmed by the presence of a new *Hin*dIII site. As the fragment used excluded the +258 site, the replacements were double mutants. This was confirmed by PCR amplification and digestion with *Sal*I.

RESULTS

As mentioned in the introduction, the first part of this study is to test the hypothesis that hyper-recombination could be observed in a cross between a PMS allele and a normal allele in close proximity, due to postmeiotic segregation of the PMS allele and repair at the normal allele. To test this hypothesis the following questions were addressed in two-point crosses involving a PMS allele and a normal allele.

1. Whether PMS alleles of all types show hyper-recombination (HR) with normal alleles.
2. Whether the HR phenomenon is distance-dependent.
3. Whether HR is associated with PMS.

In the second part of this study, a molecular model, proposed to explain the HR phenomenon, was tested with a variety of three-point crosses involving two PMS alleles and a normal allele.

For this study, the *HIS1* gene encoding N-1-(5' phospho-ribosyl) adenosine triphosphate: pyrophosphate phosphoribosyl transferase (Fink, 1964; 1965) was chosen. This locus has been intensively studied in this laboratory. The *HIS1* gene maps on the long arm of chromosome V with the closely linked markers, *HOM3* and *ARG6*, on either side. *URA3* and *TRP2* are also located on the same chromosome. The presence of well characterized and closely linked markers is advantageous since this allowed us to differentiate crossovers from non-crossovers among the intragenic recombinants. The gene has been cloned and sequenced (Hinnebusch and Fink, 1983). Many mutant alleles have also been sequenced (Savage et al., 1989). The steps involved in this study are as follows:

(i) *in vitro* construction of *his1* mutants in cloned fragment of the *HIS1* gene,

(ii) *in vivo* construction of *his1* mutants by gene replacement on the yeast chromosome using the DNA fragment containing *in vitro* constructed mutations and

(iii) genetic analysis of the diploids involving *his1* mutations.

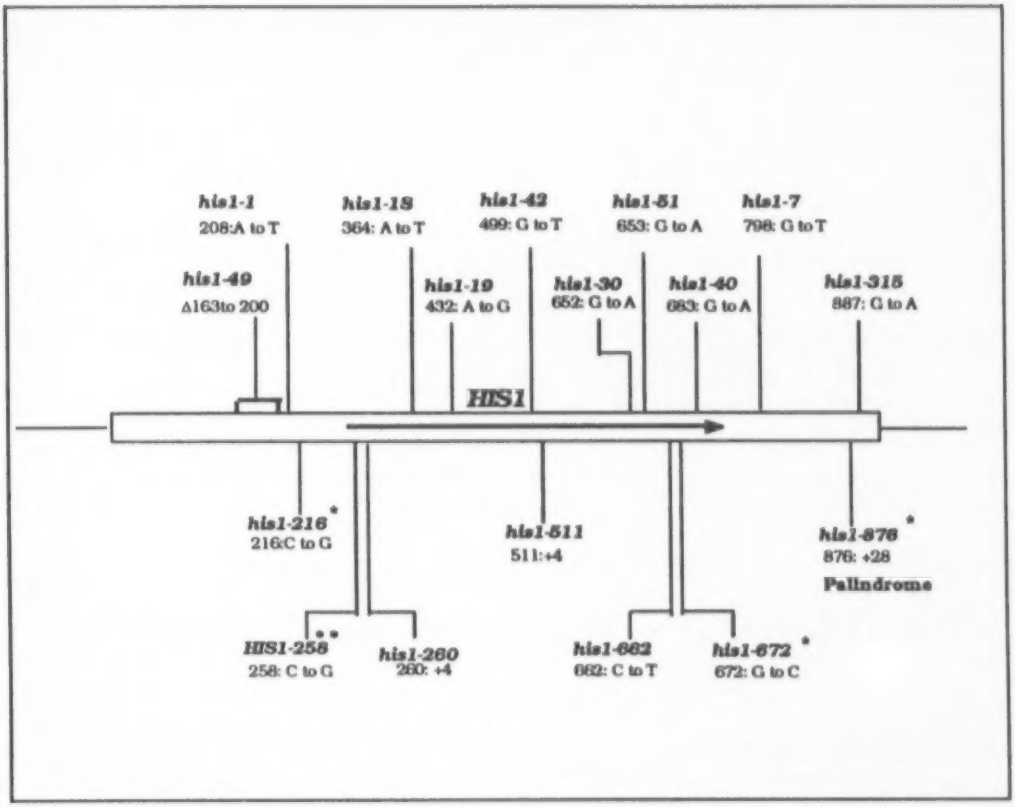

FIG. 14. Mutational changes in *his1* mutants used in this study.

* Putative PMS alleles (His^-) (see Table 3 for details).

** Putative PMS allele (His^+).

Top row: *his1* mutants already available.

Bottom row: *his1* mutants made for this study.

After *in vitro* construction and *in vivo* placement of *his1* alleles on the yeast chromosome, crosses were made with defined mutational types and distances. The outcome of recombination events both in mitosis and meiosis was studied to test the hypothesis and the molecular model.

There are two classes of *his1* alleles used in this study. One class shows high levels of postmeiotic segregation (PMS alleles) and the other, referred to here as normal alleles, which shows much lower levels of PMS (FIG. 14).

1. Newly created putative PMS alleles show postmeiotic segregation

Among the alleles created in this study, four of them are putative PMS alleles. Of the four, three are G to C transversion mutations *(his1-216, his672 and HIS1-258)*. The fourth one is a palindromic insertion. Before being used for genetic analysis, these putative PMS alleles were tested to confirm that they behave as PMS alleles. As the allele *HIS1-258* is His^+ the PMS frequency could not be calculated for this allele. Tetrad analysis for postmeiotic segregation was performed with diploids made from crossing the putative PMS allele with wild type. The frequencies of postmeiotic segregation are shown in Table 3. Thus the constructs were characterized both physically by molecular analysis and phenotypically by genetic analysis. As expected the allele *his1-662* (C to T transition mutation) did not show PMS. In this study, *his1-49* (PMS allele) was also used. It has already been shown that this allele shows 85% PMS among tetrads showing non-Mendelian segregation (Hastings, 1984).

2. Intragenic recombination at the *his1* locus in two-point crosses

Crosses were made by mating two haploids of different mating types. Five zygotes were selected for each diploid by micromanipulation as described in the Materials and Methods. After picking, the zygotes were allowed to grow on YEPD plates for 3 days to form a colony size of approximately 10^7 cells per colony. Each colony was

Table 3. Frequency of PMS among newly created *his1* alleles

his1 allele	Sequence change	Total tetrads	Aberrant segregations	PMS	PMS frequency %
his1-216	C to G	166	3	2	66
his1-672	G to C	172	3	2	66
his1-876	+28 (Palindromes)	128	4	3	75
his1-662	C to T	183	3	0	0

scooped and suspended in 2 ml YEPD liquid medium and grown to saturation. The cells were spun and washed with water. One half of the cells was plated on to MC-his and YEPD plates after appropriate dilutions to measure the spontaneous mitotic recombination rate. The mitotic recombination rate was calculated by the method of Lea and Coulson (1949). The other half of the cells was suspended in 4 ml YPA medium (presporulation medium). Growth in YPA medium improved the efficiency of sporulation. The cells were grown for 4 to 5 hours, spun down and washed with water. The cells were then suspended in water and spread on sporulation plates. After sporulation, the asci were collected from the sporulation plates and suspended in water. The spores were separated into single spores with a French pressure apparatus as described in Materials and Methods. Appropriate dilutions were plated on the YEPD medium and MC-his medium plates. Meiotic recombination frequency was calculated as frequency of prototrophs among the spores. For each cross at least 200 prototrophs were selected. The recombination frequency was standardized to frequency per base-pair for meiosis and rate per base-pair for the spontaneous mitotic rate to compare different crosses with different distances. There are four different His^- PMS alleles used in this study. The allele *HIS1-258* is a putative PMS allele but the phenotype is His^+. This allele is used only in the three-point crosses. The following are the different types of crosses made with *his1* mutants:

PMS allele X normal allele

his1-49 (deletion-PMS allele)	X	normal allele
his1-216 (C to G transversion-PMS allele)	X	normal allele
his1-672 (G to C transversion-PMS allele)	X	normal allele
his1-876 (palindromic insertion-PMS allele)	X	normal allele

Normal allele X normal allele

PMS allele X PMS allele.

The distance between the *his1* markers in the cross ranged from 8 to 687 base-pairs. These alleles are distributed throughout the *HIS1* gene. As stated earlier, the purpose of the crosses is to find out how the recombination frequency varies with distance, and thus to detect the hyper-recombination phenomenon.

A. Hyper-recombination is observed if the PMS allele is in close proximity to the normal allele

Crosses involving the *his1-49* allele

The diploids involving the *his1-49* allele include PM101 to 110 and PM120. The distance between *his1-49* and other alleles ranged from 8 to 687 bp. The closest one (*his1-1*) is located 8 bp from *his1-49* (FIG. 14). This is a transversion mutation from A to T. The recombination frequencies of the crosses involving *his1-49* are shown in Table 4 and FIG. 15. The meiotic recombination frequency per base-pair ranges from 0.2 to 4.3 x 10^{-6} except for the diploid PM120, the cross between the alleles *his1-49* and *his1-1*. In PM120 the recombination frequency is 9.4 x 10^{-5} which is two orders of magnitude higher than the frequency seen in the other *his1-49* crosses (FIG. 15). In the spontaneous mitotic recombination rate data, the same trend is noticed. The rate ranges from 0.1 to 2.2 x 10^{-8} in all the crosses except PM120 which has the highest rate (4.4 x 10^{-7}). This suggests that hyper-recombination is seen when the allele *his1-49* (a PMS allele) is close to the normal allele (*his1-1*).

Crosses involving the *his1-216* allele

The allele *his1-216* is a C to G transversion mutation and a PMS allele. The diploids made with this allele are PM130 and PM132 to PM141. This mutation is 8 bp away from *his1-1* on the right side (high conversion side). The range of distances between the two *his1* alleles in these crosses is from 8 to 671 bp (Table 5 and FIG. 16). The mitotic and meiotic recombination per base pair are shown in Table 5. The meiotic

Table 4. Mitotic and meiotic recombination (Crosses involving the *his1-49* allele)

Diploid	Relevant genotype	Distance *	Recombination	
			Mitosis**	Meiosis***
PM101	*his1-49/his1-1S*	164	0.5×10^{-8}	$3.2 \pm 0.1 \times 10^{-6}$
PM102	*his1-49/his1-19*	232	0.5×10^{-8}	$2.2 \pm 0.1 \times 10^{-6}$
PM103	*his1-49/his1-42*	299	0.3×10^{-8}	$1.9 \pm 0.4 \times 10^{-6}$
PM104	*his1-49/his1-30*	452	0.8×10^{-8}	$3.9 \pm 0.1 \times 10^{-6}$
PM105	*his1-49/his1-51*	453	2.2×10^{-8}	$1.5 \pm 0.1 \times 10^{-6}$
PM106	*his1-49/his1-40*	483	0.3×10^{-8}	$3.9 \pm 0.7 \times 10^{-6}$
PM107	*his1-49/his1-7*	598	0.3×10^{-8}	$4.3 \pm 0.9 \times 10^{-6}$
PM108	*his1-49/his1-315*	687	0.1×10^{-8}	$0.2 \pm 0.01 \times 10^{-6}$
PM109	*his1-49/his1-260*	60	0.6×10^{-8}	$2.6 \pm 0.01 \times 10^{-6}$
PM110	*his1-49/his1-511*	311	1.8×10^{-8}	$1.0 \pm 0.2 \times 10^{-6}$
PM120	*his1-49/his1-1*	8	4.4×10^{-7}	$9.4 \pm 0.2 \times 10\text{-}5$

* The distance between the two *his1* alleles in the cross in base pairs.

** Median of five independent diploids expressed as recombination rate per-base pair.

*** Mean frequency with standard deviation of five independent diploids expressed as recombination frequency per base pair.

A minimum of 200 prototrophs was studied for each cross both in mitosis and meiosis.

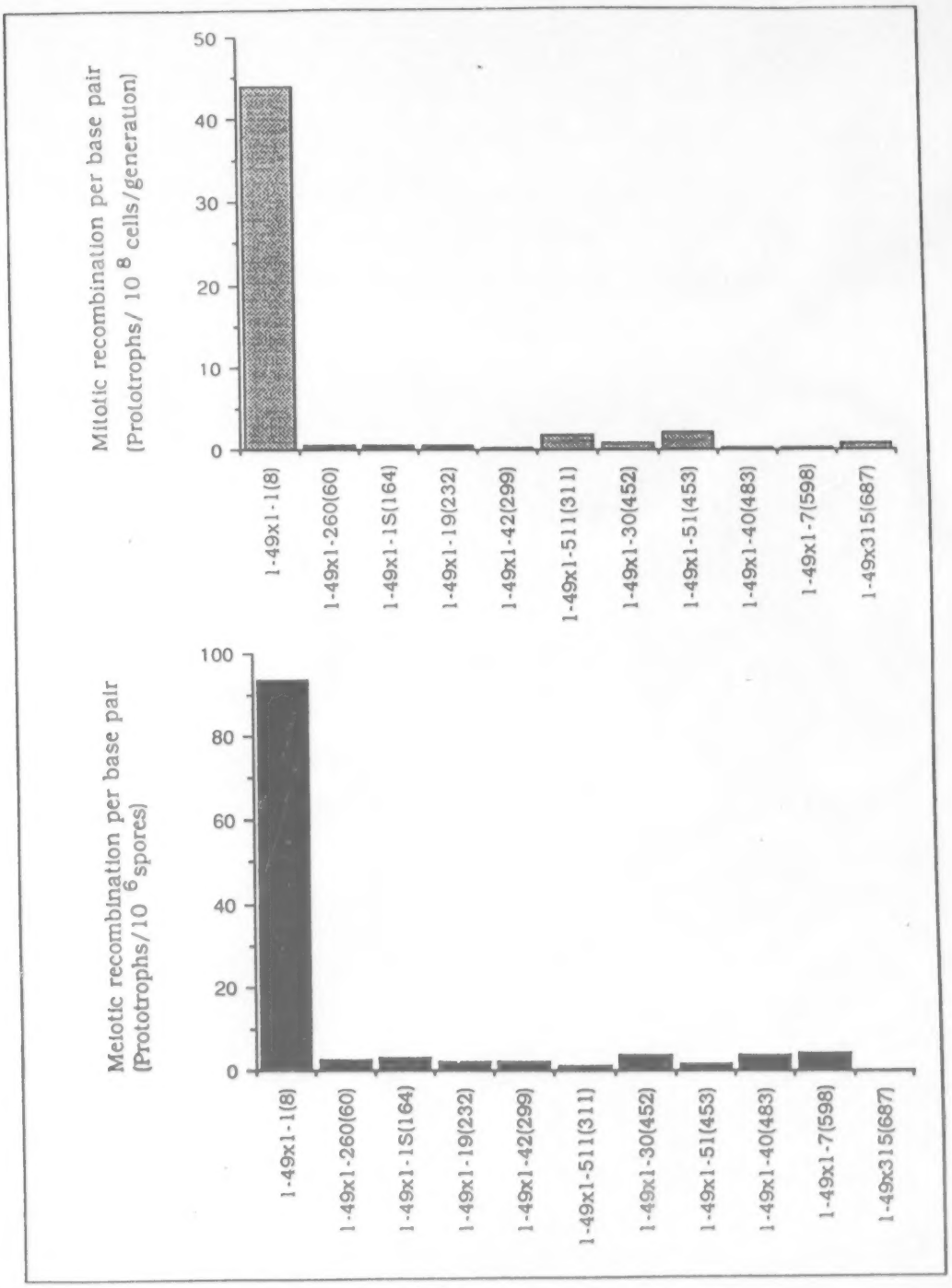

FIG. 15. Mitotic and meiotic recombination in crosses involving the *his1-49* allele.

The distance between alleles (bp) in each cross is indicated in parentheses.

Table 5. Mitotic and meiotic recombination (Crosses involving the *his1-216* allele)

Diploid	Genotype	Distance *	Recombination	
			Mitosis**	Meiosis ***
PM130	*his1-216/his1-1*	8	7.4×10^{-7}	$5.1 \pm 0.4 \times 10^{-5}$
PM132	*his1-216/his1-1S*	148	3.3×10^{-8}	$0.8 \pm 0.1 \times 10^{-6}$
PM133	*his1-216/his1-19*	216	1.9×10^{-8}	$1.3 \pm 0.3 \times 10\text{-}6$
PM134	*his1-216/his1-42*	283	3.0×10^{-8}	$1.6 \pm 0.2 \times 10\text{-}6$
PM135	*his1-216/his1-30*	436	0.5×10^{-8}	$1.4 \pm 0.2 \times 10\text{-}6$
PM136	*his1-216/his1-51*	437	3.3×10^{-8}	$1.7 \pm 0.2 \times 10\text{-}6$
PM137	*his1-216/his1-7*	582	0.2×10^{-8}	$1.0 \pm 0.1 \times 10\text{-}6$
PM138	*his1-216/his1-315*	671	0.7×10^{-8}	$1.2 \pm 0.8 \times 10\text{-}6$
PM139	*his1-216/his1-40*	467	0.2×10^{-8}	$1.7 \pm 0.1 \times 10^{-6}$
PM140	*his1-216/his1-260*	44	1.6×10^{-8}	$5.2 \pm 0.4 \times 10^{-6}$
PM141	*his1-216/his1-511*	295	0.4×10^{-8}	$1.4 \pm 0.1 \times 10^{-6}$

* The distance between the two *his1* alleles in the cross in base pairs.

** Median of five independent diploids expressed as recombination rate per-base pair.

*** Mean frequency with standard deviation of five independent diploids expressed as recombination frequency per base pair.

A minimum of 200 prototrophs was studied for each cross both in mitosis and meiosis.

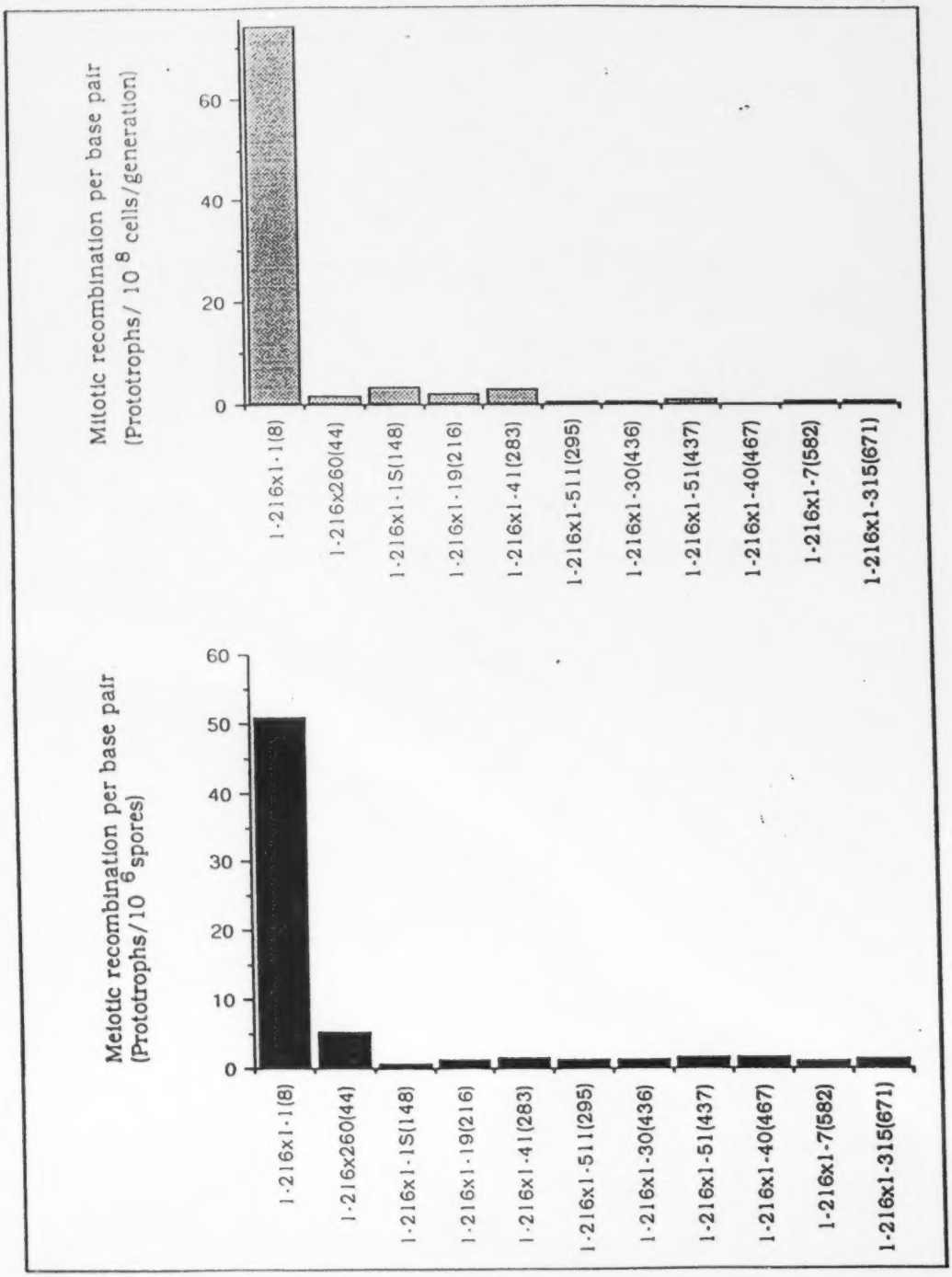

FIG. 16. Mitotic and meiotic recombination in crosses involving the *his1-216* allele. The distance between alleles (bp) in each cross is indicated in parentheses.

recombination frequency is highest (5.1 x 10^{-5}/bp) in the cross PM130 in which the distance between two alleles is 8 bp. The other crosses showed the normal frequency of recombination which ranged from 0.8 to 5.2 x 10^{-6} in meiosis. The mitotic data also showed the same trend (FIG. 16). This set of results again indicates that hyper-recombination can be observed between a PMS allele and a normal allele in close proximity.

Crosses involving the *his1-672* allele

The allele *his1-672* is a G to C transversion mutation. In the heteroduplex the mismatches formed will be same as in *his1-216*. The distance between the alleles in the crosses range from 11 to 464 bp. The crosses involving *his1-672* are PM150 to 155 and PM157. The diploids PM150, PM151 and PM152 have the normal allele (non-PMS allele) within a short distance on either side of *his1-672*. In the diploid PM150, *his1-40* is located on the right side (3' side) of *his 1-672* and in PM151 (*his1-51*) and 152 (*his1-30*) the normal allele is located on the left side (5' side) (FIG. 14). Recombination in crosses involving *his 1-672* are shown in Table 6. The crosses which have the alleles within a short distance (PM150, PM151 and PM152) show hyper-recombination in both mitosis and meiosis (FIG. 17). This result shows that the elevated recombination is seen on both sides of a PMS allele (5' or 3' side) and suggests that there is no polarity difference in the HR phenomenon.

Crosses involving the *his1-876* allele

The mutation *his1-876* is a palindromic insertion which is located close to the 3' end of the *HIS1* gene. The closest allele, *his1-315*, is located at the extreme 3' end (at +887) of the gene and all other alleles are located to the left of this allele (FIG. 14). The crosses involving this mutant are PM180 to 184 and PM189. The HR phenomenon is noticed both in mitosis and meiosis (Table 7) in the diploid PM180, which has the alleles 11 bp apart, but not in other crosses where the distances are greater (FIG. 18).

Table 6. Mitotic and meiotic recombination (Crosses involving the *his1-672* allele)

Diploid	Relevant genotype	Distance *	Recombination	
			Mitosis**	Meiosis***
PM150	*his1-672/his1-40*	11	6.2×10^{-7}	$12.2 \pm 0.8 \times 10^{-5}$
PM151	*his1-672/his1-51*	19	5.2×10^{-7}	$9.5 \pm 0.4 \times 10^{-5}$
PM152	*his1-672/his1-30*	20	4.7×10^{-7}	$9.3 \pm 0.8 \times 10^{-5}$
PM153	*his1-672/his1-7*	126	2.3×10^{-8}	$4.7 \pm 0.9 \times 10^{-6}$
PM154	*his1-672/his1-315*	215	1.5×10^{-8}	$2.4 \pm 0.1 \times 10^{-6}$
PM155	*his1-672/his1-1*	464	1.0×10^{-8}	$2.1 \pm 0.03 \times 10^{-6}$

* The distance between two *his1* alleles in the cross in base pairs.

** Median of five independent diploids expressed as recombination rate per base pair.

*** Mean frequency with standard deviation of five independent diploids expressed as recombination frequency per base pair.
A minimum of 200 prototrophs was studied for each cross both in mitosis and meiosis.

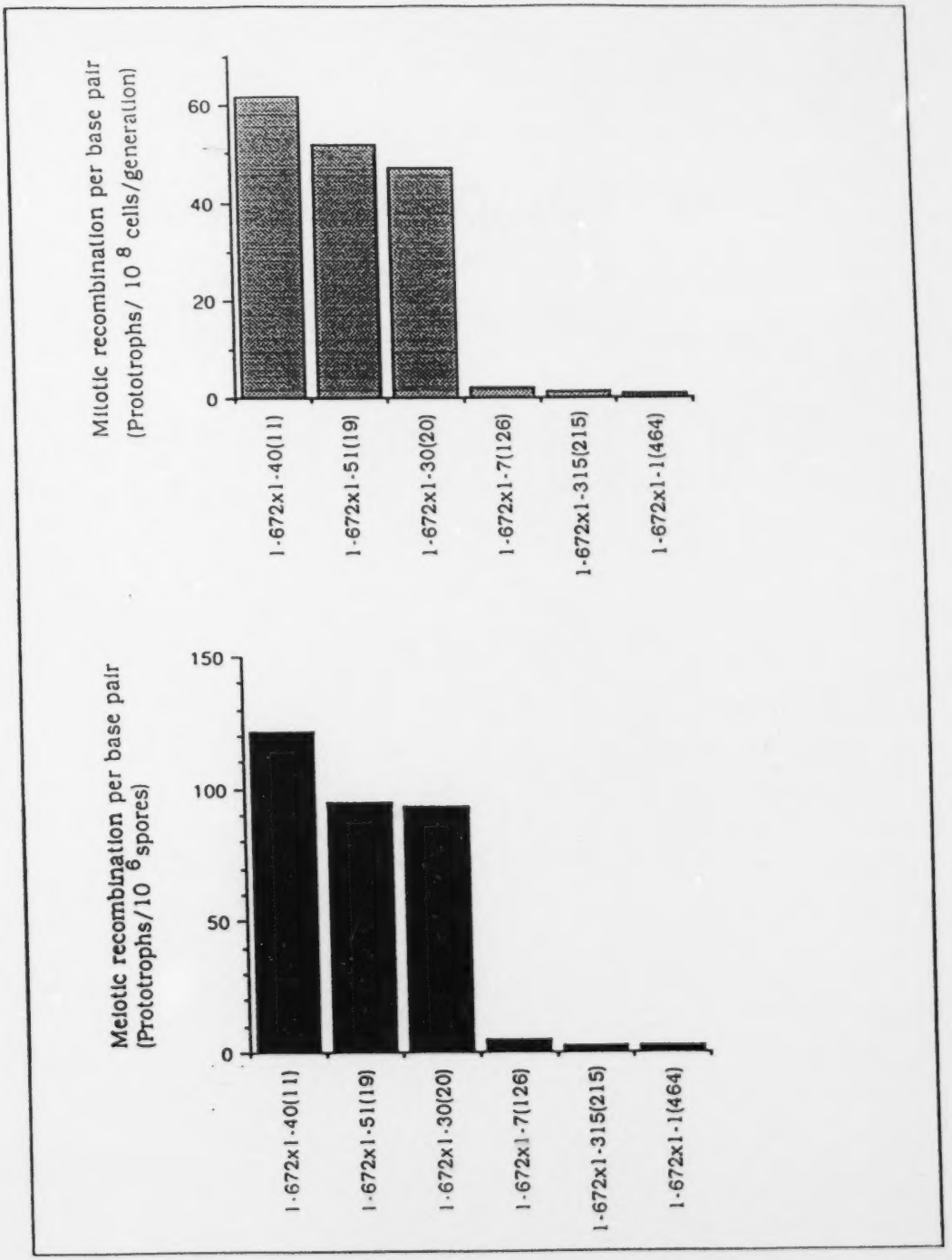

FIG. 17. Mitotic and meiotic recombination in crosses involving the *his1-672* allele. The distance between alleles (bp) in each cross is indicated in parentheses.

Table 7. Mitotic and meiotic recombination (Crosses involving the *his1-876* allele)

Diploid	Relevant genotype	Distance *	Recombination	
			Mitosis**	Meiosis***
PM 180	*his1-876/his1-315*	11	6.1×10^{-7}	$9.1 \pm 1.1 \times 10^{-5}$
PM181	*his1-876/his1-7*	78	1.4×10^{-8}	$1.5 \pm 0.3 \times 10^{-6}$
PM182	*his1-876/his1-40*	193	2.8×10^{-8}	$3.3 \pm 0.5 \times 10^{-6}$
PM 183	*his1-876/his1-42*	377	1.9×10^{-8}	$0.9 \pm 0.06 \times 10^{-6}$
PM184	*his1-876/his1-30*	224	1.1×10^{-8}	$1.9 \pm 0.3 \times 10^{-6}$
PM 189	*his1-876/his1-1*	668	2.4×10^{-8}	$0.6 \pm 0.05 \times 10^{-6}$

* The distance between two *his1* alleles in the cross in base pairs.

** Median of five independent diploids expressed as recombination rate per base pair.

*** Mean frequency with standard deviation of five independent diploids expressed as recombination frequency per base pair.

A minimum of 200 prototrophs was studied for each cross both in mitosis and meiosis.

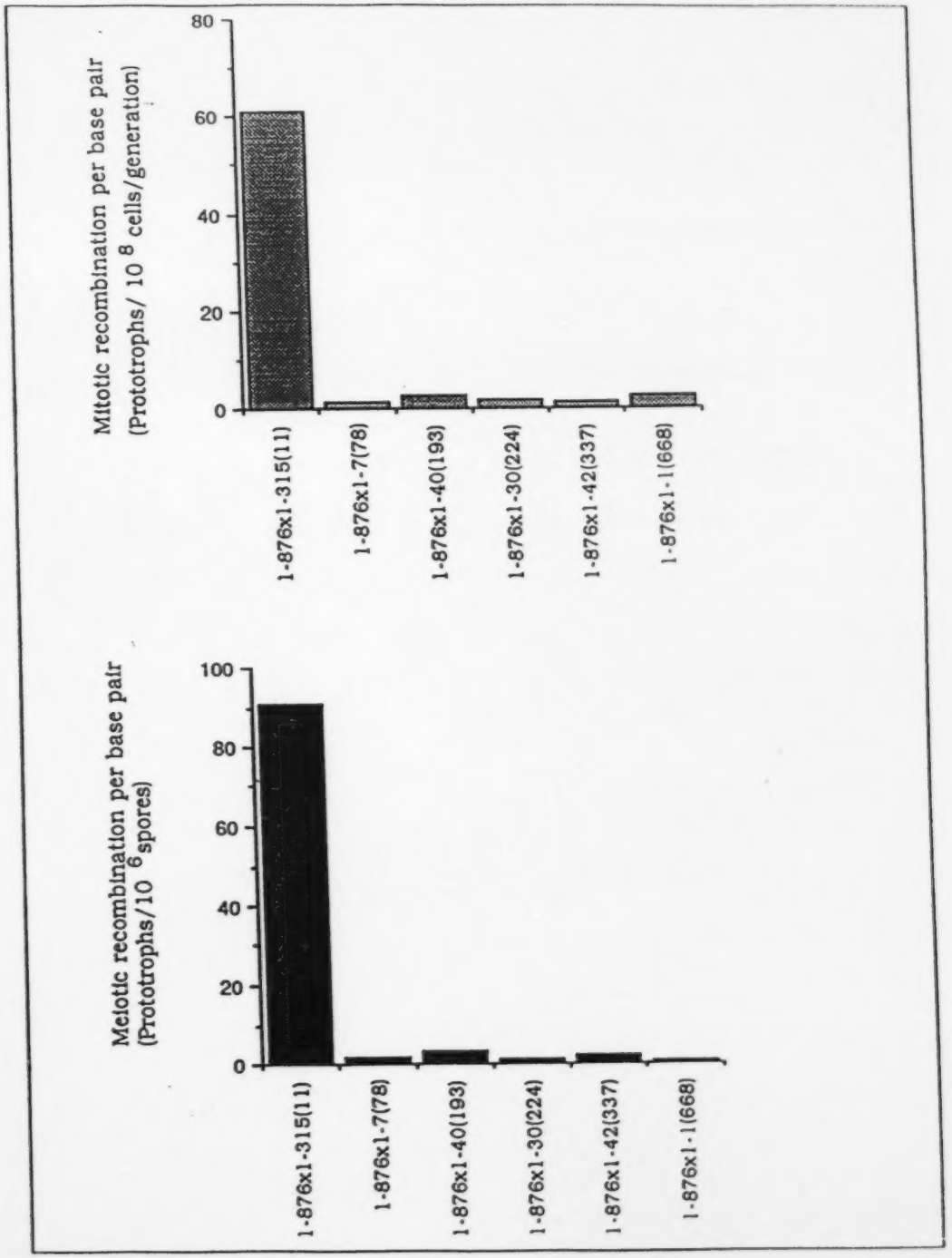

FIG. 18. Mitotic and meiotic recombination in crosses involving the *his1-876* allele. The distance between alleles (bp) in each cross is indicated in parentheses.

Crosses involving the normal alleles

These crosses serve as controls for the above crosses (PMS allele X normal allele). In these crosses the range of distance between two alleles is from 9 to 590. The crosses involving *his1-662* are particularly important. In those crosses the alleles are located on either side of the *his1-662* allele, as in the crosses with *his1-672*. There is no spectacular difference in the recombination frequency among these control crosses even for the small distances (FIG. 19). This can be seen in four crosses, three involving *his1-662* (PM190, PM191 and PM192) and the other involving *his1-511* and *his1-42* (PM178) (Tables 8 and 9).

Among all the crosses recombination is very high in the crosses PM120, PM130, PM150, PM151, PM152 and PM180. HR is seen if the PMS allele is close to the normal allele. In these crosses the distances between PMS allele and the other allele ranges from 8 to 20 base-pairs. HR is observed on both sides of PMS alleles. The allele *his1-876* is located at the extreme 3' end of the gene and *his1-49* is located at the 5' end, but both of them showed hyper-recombination. Therefore, the hyper-recombination phenomenon is not position-specific. The control crosses with normal alleles within the same distances showed recombination frequencies which are orders of magnitude lower than those crosses involving a PMS allele. From these results, the studies of *cyc1* mutants (Moore et al., 1989) and experiments with the *HIS4* gene (Detloff et al., 1992) it can be generalized that the hyper-recombination phenomenon is not specific to a particular gene but a general phenomenon attributed to this type of cross involving a PMS allele and a normal allele within a short distance (8 to 26 bp) (FIG. 15 to 18).

B. Crosses involving two PMS alleles

Crosses were also made between different pairs of PMS alleles different distances apart. The distances vary from 16 to 676 bp (Table 10). As the repair

Table 8. Mitotic and meiotic recombination (Crosses involving normal alleles)

Diploid	Relevant genotype	Distance *	Recombination	
			Mitosis**	Meiosis***
PM121	*his1-1/his1-40*	475	1.0×10^{-8}	$1.9 \pm 0.4 \times 10^{-6}$
PM122	*his1-1/his1-7*	590	1.5×10^{-8}	$3.6 \pm 0.8 \times 10^{-6}$
PM123	*his1-1/his1-1S*	156	1.7×10^{-8}	$2.9 \pm 0.4 \times 10^{-6}$
PM124	*his1-1/his1-30*	444	0.5×10^{-8}	$3.7 \pm 0.5 \times 10^{-6}$
PM129	*his1-1/his1-511*	303	2.3×10^{-8}	$1.4 \pm 0.5 \times 10^{-6}$
PM170	*his1-30/his1-7*	146	1.6×10^{-8}	$3.3 \pm 0.4 \times 10^{-6}$
PM171	*his1-7/his1-40*	115	2.8×10^{-8}	$2.3 \pm 0.3 \times 10^{-6}$
PM173	*his1-30/his1-40*	31	5.1×10^{-8}	$1.4 \pm 0.1 \times 10^{-6}$
PM175	*his1-7/his1-260*	538	0.3×10^{-8}	$1.1 \pm 0.08 \times 10^{-6}$
PM177	*his1-260/his1-511*	251	4.6×10^{-8}	$1.9 \pm 0.2 \times 10^{-6}$
PM178	*his1-511/his1-42*	12	1.8×10^{-8}	$1.1 \pm 0.2 \times 10^{-6}$
PM179	*his1-511/his1-19*	79	1.5×10^{-8}	$3.4 \pm 0.9 \times 10^{-6}$

* The distance between two *his1* alleles in the cross.

** Median of five independent diploids expressed as recombination rate per base pair.

*** Mean frequency with standard deviation of five independent diploids expressed as recombination frequency per base pair.

A minimum of 200 prototrophs was studied for each cross both in mitosis and meiosis.

Table 9. Mitotic and meiotic recombination (Crosses involving the *his1-662* allele)

Diploid	Relevant genotype	Distance *	Recombination	
			Mitosis**	Meiosis***
PM 191	*his1-662/his1-51*	9	0.9×10^{-8}	$1.9 \pm 0.06 \times 10^{-6}$
PM192	*his1-662/his1-30*	10	0.8×10^{-8}	$1.4 \pm 0.1 \times 10^{-6}$
PM 190	*his1-662/his1-40*	21	0.4×10^{-8}	$1.3 \pm 0.09 \times 10^{-6}$
PM 193	*his1-662/his1-42*	163	1.2×10^{-8}	$2.2 \pm 0.1 \times 10^{-6}$
PM 195	*his1-662/his1-315*	225	0.9×10^{-8}	$1.2 \pm 0.2 \times 10^{-6}$

* The distance between two *his1* alleles in the cross in base pairs.

** Median of five independent diploids
expressed as recombination rate per base pair.

*** Mean frequency with standard deviation of five independent diploids
expressed as recombination frequency per base pair.
A minimum of 200 prototrophs was studied for each cross both in mitosis and meiosis.

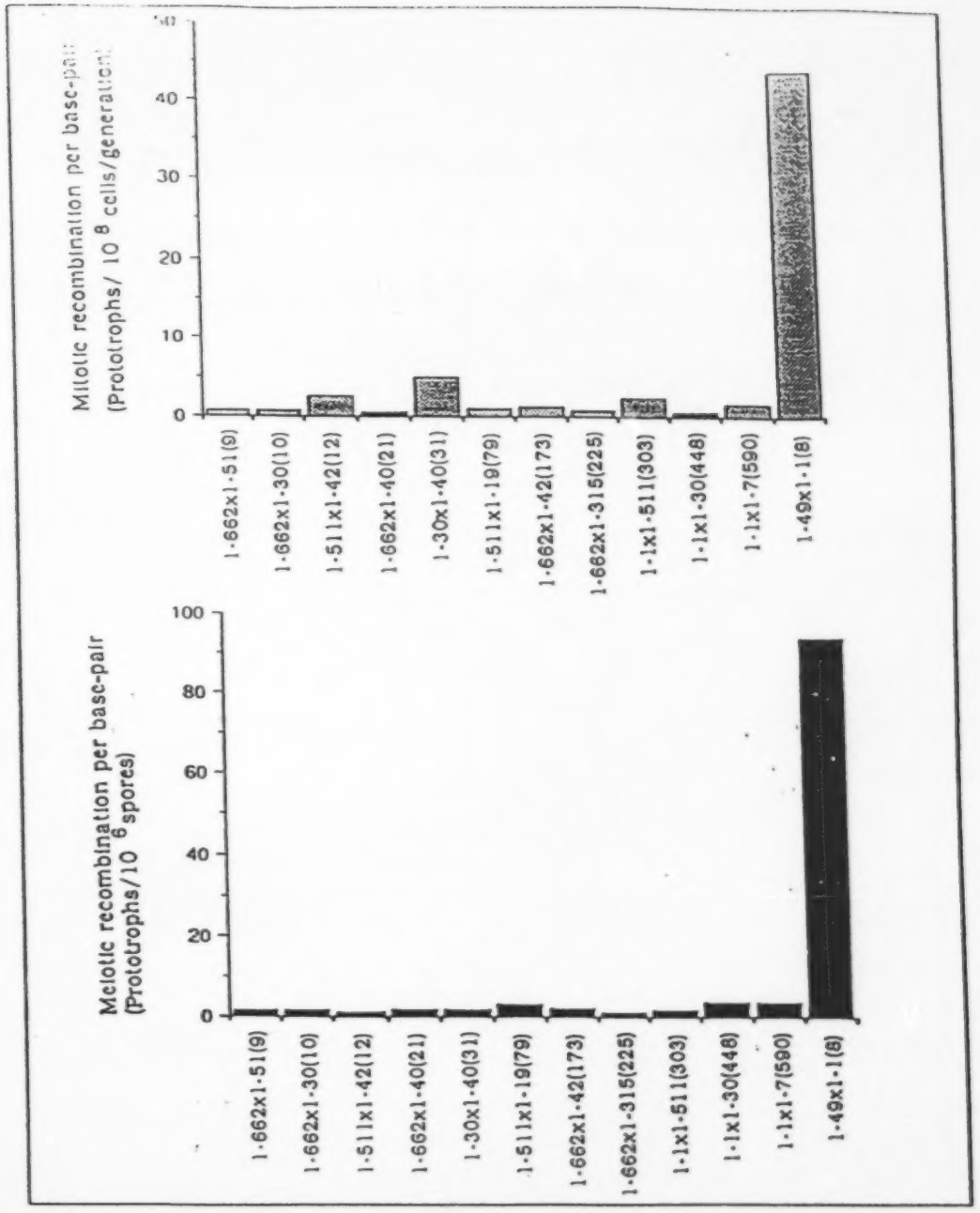

FIG. 19. Mitotic and meiotic recombination in crosses involving normal alleles. The distance (bp) between alleles in each cross is indicated in parentheses. A HR cross *(his1-49 x his1-1)* is included for comparision.

Table 10. Mitotic and meiotic recombination (Crosses involving two PMS alleles)

Diploid	Relevant genotype	Distance *	Recombination	
			Mitosis**	Meiosis***
PM 131	*his1-49/his1-216*	16	7.3×10^{-7}	$8.30 \pm 0.4 \times 10^{-5}$
PM142	*his1-216/his1-672*	456	0.9×10^{-8}	$1.1 \pm 0.05 \times 10^{-6}$
PM 157	*his1-49/his1-672*	472	0.6×10^{-8}	$1.4 \pm 0.1 \times 10^{-6}$
PM 185	*his1-49/his1-876*	676	1.1×10^{-8}	$0.7 \pm 0.1 \times 10^{-6}$
PM 186	*his1-672/his1-876*	204	0.9×10^{-8}	$2.3 \pm 0.2 \times 10^{-6}$
PM 187	*his1-2 16/his1-876*	660	0.8×10^{-8}	$1.1 \pm 0.08 \times 10^{-6}$

* The distance between two *his1* alleles in the cross.

** Median of five independent diploids expressed as recombination rate per base pair.

*** Mean frequency with standard deviation of five independent diploids expressed as recombination frequency per base pair.

A minimum of 200 prototrophs was studied for each cross both in mitosis and meiosis.

efficiency is low in the PMS alleles, these crosses are expected to show low levels of recombination. Recombination in these crosses is very low except in the cross involving *his1-49* X *his1-216* (PM131). This suggests that one of the alleles in that cross may act like a normal allele. A reason for this is discussed later.

Spontaneous reversion frequencies of *his1* alleles

The spontaneous reversion frequency of all the *his1* mutants involved in this study was calculated by the method of Lea and Coulson (1949) using mini-fluctuation tests (Table 11). Five logarithmically growing colonies were picked from YEPD medium, suspended in 2 ml of YEPD and incubated with shaking at 30°C for four days. The growth of each clone was considered to be from one cell. Hence the median number of mutants per plate could be directly transformed to mutation rate per cell per generation, without having to consider initial cell or initial revertant numbers, by using the method of the median (Lea and Coulson, 1949). The reversion frequency is highest for the allele *his1-7* and lowest for the allele *his1-315*. The reversion frequency for the allele *HIS1-258* was not determined, since this is a silent mutation and the phenotype is His^+. The reversion frequency of *his1* alleles is allele-specific and not position-specific. For example *his1-30* and *his1-51* are adjacent alleles at positions +652 and +653, respectively, yet there is a considerable difference in the reversion rate between these two alleles. As expected, there is no reversion of *his1-49*, since this is a deletion mutation. The allele *his1-876* is a perfect palindromic insertion. Reversion could occur if the palindrome is perfectly excised. There was no reversion observed among 10^{10} cells (Table 11). As the reversion rate for every allele is far lower than the mitotic rate and meiotic recombination frequency, it will not have any effect on the outcome of either mitotic or meiotic recombination.

Table 11. Reversion rate of *his1* mutants

his1 mutants	Reversion rate*	
	a	α
his1-1	1.9×10^{-9}	4.4×10^{-9}
his1-7	4.9×10^{-7}	3.8×10^{-7}
his1-1S	7.7×10^{-9}	7.4×10^{-9}
his1-19	8.3×10^{-9}	3.6×10^{-9}
his1-30	1.6×10^{-7}	1.8×10^{-7}
his1-40	9.9×10^{-9}	1.8×10^{-8}
his1-42	4.1×10^{-9}	4.7×10^{-9}
his1-49	0	0
his1-51	5.7×10^{-9}	8.5×10^{-9}
his1-216	NC	1.2×10^{-9}
his1-315	7.5×10^{-9}	2.1×10^{-9}
his1-662	NC	3.9×10^{-7}
his1-672	NC	6.6×10^{-8}
his1-876	NC	$<10^{-10}$

* Reversion rate per generation per cell.

NC: Not calculated.

C. Postmeiotic segregation is observed in hyper-recombinants

We supposed that hyper-recombination is due to postmeiotic segregation of the PMS allele and repair at the normal allele. This was tested in the diploid PM130. This is a cross between *his1-216 x his1-1* (PMS allele x normal allele). After sporulation, the spores were separated by treatment with Glusulase and passage through a French pressure apparatus. The random spores were plated on YEPD plates. The dilutions were made such that 50 to 60 colonies grew on each plate. The colonies were replica-plated onto MC-his medium plates. Among 9528 spore colonies 6 prototrophs were observed. Among the prototrophs two of them very clearly showed postmeiotic segregation. It is possible that some or all of the four prototyphe-containing colonies also show PMS which could not be seen because of overgrowth of the His^- sections by His^+ cells. The genotypes of these prototrophs are shown in Table 12. The postulate was also tested in unselected tetrads by dissecting the tetrads with a micro-manipulator. The same diploid (PM130) was used to test this. Based on the random spore analysis one or two PMS events are expected out of 1000 tetrads. One prototroph was observed out of 247 tetrads (Table 13). This single prototroph also showed PMS. Both results support the hypothesis that prototrophy arises from PMS events.

D. Flanking marker configuration of *his1* recombinant spores

The prototrophs can be classified as parental types (non-crossovers) and recombinant types (crossovers). There are four possible configurations of the flanking markers, *hom3* and *arg6*, which are associated with *his1* recombinants. They are P1 and P2 (parental classes); R1 and R2 (recombinant classes). P1 refers to the parental class prototrophs showing conversion of the marker proximal to the high conversion end of the polarity gradient and P2 refers to the prototrophs showing conversion of markers distal to the high conversion end of the polarity gradient. The frequency of the P1 class is higher than the P2 class in a series of two-point crosses (for details please

Table 12. Genotypes of prototrophs identified by screening from random spores from the diploid PM130 (*his1-216 X his1-1*)

Prototroph No	HOM3	HIS1	ARG6	TRP2
1	-	- / +	-	-
2	+	- / +	-	-
3	-	+	-	-
4	+	+	+	+
5	+	+	+	+
6	+	+	-	-

\+ Prototrophy for that marker.

_ Auxotrophy for that marker.

+/- Postmeiotic segregation of that marker.

Relevant genotype $\frac{HOM3\ his1\text{-}1\ ARG6\ TRP2}{hom3\ his1\text{-}216\ arg6\ trp2}$

Table 13. Genotypes of spores from an unselected tetrad from the diploid PM130 (*his1-216 X his1-1*) which showed PMS

Spore No	HOM3	HIS1	ARG6	TRP2
A	+	-	+	+
B	+	-	-	-
C	-	- / +	+	+
D	-	-	-	-

\+ Prototrophy for that marker.

_ Auxotrophy for that marker.

+/- Postmeiotic segregation of that marker.

Relevant genotype $\frac{\underline{HOM3\ his1\text{-}1\ ARG6\ TRP2}}{hom3\ his1\text{-}216\ arg6\ trp2}$

refer to the review of Hastings 1988). The data from two-point crosses in this study also show that the P1 class is greater than the P2 class as expected (Table 14) except in the crosses PM130, PM151 and PM152 in which P2>P1 (Table 15). In those crosses, the PMS alleles are located at the high conversion end. This shows that heteroduplex resolution in HR crosses may be different from the crosses showing normal level of recombination.

It is interesting to note that the P1 class of prototrophs shows conversion of alleles at the 3' end of the gene and thus the polarity gradient of gene conversion is from 3' to 5'. This type of polarity gradient was inferred from the sequencing data of *his1* mutants (Savage et al., 1989). My two-point cross data provide a direct demonstration of the polarity gradient. This type of polarity gradient is also seen for the *HIS3* locus of yeast (please refer to the review section for details).

3. Analysis of three-point crosses involving two PMS alleles and a normal allele

Based on the results, a molecular model was proposed to account for the HR phenomenon. In the model, we propose that the HR phenomenon is due to blockage of mismatch repair at the PMS allele and unidirectional repair of the normal allele (for further details please see the discussion). I wished to test the hypothesis that a PMS allele close to a normal allele could block a repair tract extending towards it from the normal allele. This hypothesis was tested by placing two PMS alleles on one side of a normal allele so that the repair of the normal allele may be blocked by the proximal PMS allele thereby preventing co-correction of the distal PMS allele. As a control the normal allele was placed between two PMS alleles. The strategy for making the crosses is shown in FIG. 20. As seen earlier, double mutants were made by gene replacement (FIG. 13). For this purpose the strain which already has one mutation was used so that after replacement one more mutation is introduced. The PMS alleles *HIS1-258* and *his1-672* are common to all the three-point crosses. Only the normal alleles (*his1-30*

Table 14. Parental and recombinant classes of prototrophs (percent) from the crosses showing normal recombination

Diploid	cross	Parental		Recombinant	
		P1	P2	R1	R2
PM101	*his1-49/his-1S*	56	10	28	6
PM102	*his1-49/his1-19*	41	17	31	11
PM103	*his1-49/his1-42*	43	23	28	6
PM104	*his1-49/his1-30*	49	17	31	3
PM133	*his1-216/his1-19*	63	5	21	11
PM135	*his1-216/his1-30*	46	18	21	15
PM153	*his1-672/his1-7*	40	20	26	14
PM155	*his1-672/his1-1*	30	18	42	10
PM181	*his1-876/his1-7*	21	12	47	20
PM183	*his1-876/his1-42*	51	9	35	5
PM121	*his1-1/his1-40*	30	18	40	12
PM122	*his1-1/his1-7*	34	25	26	15
PM170	*his1-30/his1-7*	43	16	33	8
PM191	*his1-662/his1-51*	32	29	31	8
PM192	*his1-662/his1-30*	33	30	22	15
PM193	*his1-662/his1-42*	50	8	33	9

P1 Parental class of prototrophs showing conversion of allele at 3' side (high conversion side).

P2 Parental class of prototrophs showing conversion of allele at 5' side (low conversion side).

A minimum of 200 prototrophs was studied for each cross.

Table15. Parental and recombinant classes of prototrophs (percent) from the crosses showing hyper-recombination

Diploid	cross	Parental		Recombinant	
		P1	P2	R1	R2
PM120	*his1-49/his1-1*	22	21	22	35
PM130	*his1-216/his1-1*	13	46	24	17
PM131	*his1-49/his1-216*	45	10	40	5
PM150	*his1-672/his1-40*	41	28	20	11
PM151	*his1-672/his1-51*	18	31	27	24
PM152	*his1-672/his1-30*	27	33	21	19
PM180	*his1-876/his1-315*	67	3	27	4
PM200	*his1-258,40/his1-672*	35	12	32	21
PM201	*his1-258,30/his1-672*	18	27	30	25
PM300	*his1-258,672/his1-40*	25	27	24	24
PM301	*his1-258,672/his1-30*	38	21	32	9

P1 Parental class of prototrophs showing conversion of allele at 3' side (high conversion side).

P2 Parental class of prototrophs showing conversion of allele at 5' side (low conversion side).

A minimum of 200 prototrophs was studied for each cross.

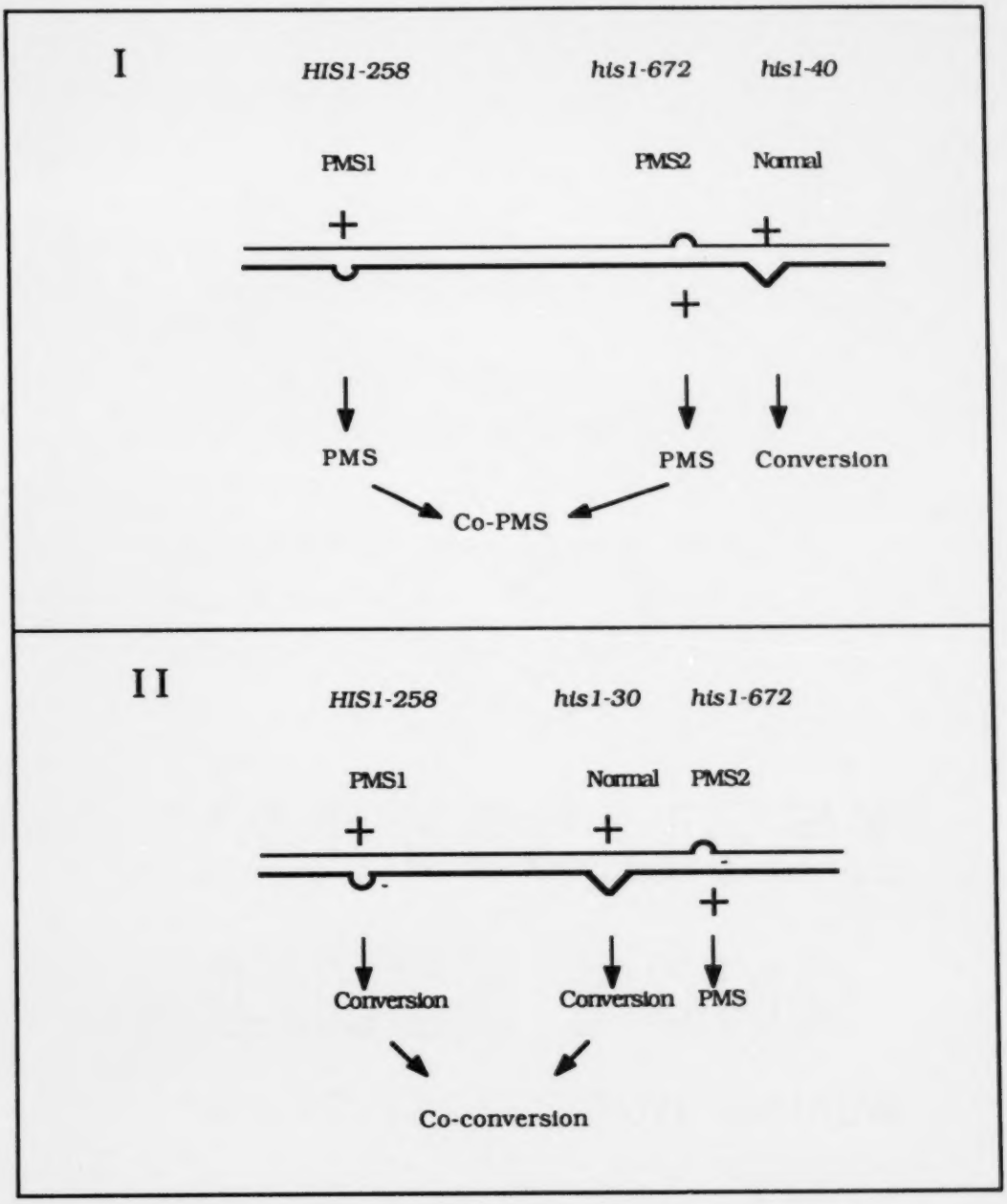

FIG. 20. Three point crosses involving two PMS alleles and a normal allele.

I. Normal allele is located on the high conversion end.

II. Normal allele is located between two PMS alleles.

and *his1-40*) are different. The allele *HIS1-258* has no phenotype but can be identified by the absence of a *SalI* site at +258 of the *HIS1* gene. The phenotype of *his1-672* is His^-. The results of these crosses follow:

His1-258,40 X his1-672 (PM200)

In this cross, two PMS alleles were placed on one side (the low conversion side (FIG. 20). The haploid with the double mutation (*his-1-258,40*) was made by replacing the wild-type sequence with a *HIS1-258* mutant fragment in the strain carrying the *his1-40* mutation (FIG. 13). This haploid was crossed to another haploid having the *his1-672* mutation to get the three-point cross. As *HIS1-258* has no phenotype, this cross is expected to show HR due to the short distance between the normal allele (*his1-40*) and the PMS allele (*his1-672*). The frequency of recombination in meiosis and rate in mitosis are very high when compared to control crosses (Table 16). As *his1-40* and *HIS1-258* alleles are in *cis*, normally the *HIS1-258* would be co-corrected by this allele. Co-correction will reintroduce the *SalI* site at +258. According to our model we propose that repair in the normal allele is restricted unidirectionally by the nearby PMS allele (*his1-672*) so that the *HIS1-258* allele is not co-corrected (FIG. 20). The postulate was tested by picking the parental class prototrophic spore colonies and analyzing them by PCR amplification of genomic DNA and restriction with *SalI*. Among the fourteen P1 class prototrophs tested, none of them showed co-correction. Most of the P2 prototrophs (2/12) also did not show correction of *HIS1-258* with *his1-40*. The flanking marker configurations show that P1 is greater than P2 for the diploid PM200 (Table 15). This is expected since *his1-40* is on the right side (high conversion side) of the other two alleles.

His1-258,30 X his1-672 (PM201)

In the diploid PM201, the normal allele (*his1-30*) was placed between two PMS alleles (FIG. 20). The haploid with two mutations was made by replacing the wild-type

Table 16. Mitotic and meiotic recombination (three-point crosses)

Diploid	Relevant genotype	Distance *	Recombination	
			Mitosis**	Meiosis***
PM 200	*his1-258,40/his1-672*	11	2.0×10^{-7}	$9.3 \pm 0.5 \times 10^{-5}$
PM 201	*his1-258,30/ 1-672*	20	3.7×10^{-7}	$3.7 \pm 0.4 \times 10^{-5}$
PM 300	*his1-258,672/his1-40*	11	3.6×10^{-7}	$6.2 \pm 0.7 \times 10^{-5}$
PM301	*his1-258,672/his1-30*	20	4.9×10^{-7}	$2.8 \pm 0.2 \times 10^{-5}$
PM303	*his1-258,672/his1-7*	126	0.8×10^{-8}	$1.3 \pm 0.2 \times 10^{-7}$

* The distance between two *his1* alleles in the cross in base pairs.

** Median of five independent diploids expressed as recombination rate per base pair.

*** Mean frequency with standard deviation of five independent diploids expressed as recombination frequency per base pair.
A minimum of 200 prototrophs was studied for each cross both in mitosis and meiosis.

sequence (FIG. 13). This double mutant was then crossed to a haploid strain having the *his1-672* allele to get the diploid PM201. As the distance between the normal allele (*his1-30*) and one PMS allele (*his1-672*) is short, this cross is also expected to give HR. The elevated recombination is seen both in mitosis and meiosis as predicted (Table 16). As the normal allele (*his1-30*) in the diploid PM201 is on the other side of the PMS allele (*his1-672*), our hypothesis predicts that the normal allele will co-correct the other PMS allele (*HIS1-258*) as shown in FIG. 20. Co-correction of *HIS1-258* with *his1-30* can be identified by the presence of a *SalI* site at +258. P1 and P2 class prototrophs were analyzed by PCR amplification of the genomic DNA followed by *SalI* restriction. Of thirteen P2 class prototrophs tested, seven of them showed co-correction of *his1-30* and *HIS1-258*. Most of the P1 class prototrophs showed co-correction (9/14). This shows that *HIS1-258* is co-corrected if the normal allele repair tract is not obstructed. This cross shows P2>P1 (Table 15). This is similar to the result obtained from the diploid PM151(*his1-672 x his1-30*).

His1-258,672 x his1-40 (PM300) and *his1-258,672 x his1-30* (PM301)

In another set of crosses PM300 and PM301 the PMS alleles were placed in *cis*. A haploid with two mutations was constructed in two steps as shown previously (FIG. 13) and mated to haploids having either the *his-40* or the *his1-30* mutation to get three-point cross diploids PM300 and PM301, respectively. The mutations in the PMS alleles *HIS1-258* (G to C) and *his1-672* (C to G) are of opposite transversion types. If a G/G mismatch is formed at one PMS allele a C/C is formed at another allele. As a G/G mismatch is repaired well, it is expected to co-correct the other alleles and hence this cross is expected to produce a normal level of recombination. Unexpectedly, both diploids showed HR (Table 16). The parental class of prototrophs show that P2>P1 in PM300 and P1>p2 in the diploid PM301 (Table 15). The possible reasons for the above results are presented in the discussion.

DISCUSSION AND CONCLUSION

1. The hyper-recombination phenomenon (HR) is due to proximity of the PMS allele to the normal allele

The results in the preceding section show that crosses involving a PMS allele and a normal allele show HR if the distance between two alleles is short. Similar crosses involving two normal alleles with the same distance do not show HR (FIG. 15 to 18). The study of *cyc1* mutants (Moore et al., 1988) also showed elevated recombination in a cross between an allele with a G to C transversion and an allele with any other type of mutation. The HR effect is seen in the *CYC1* locus when two mutational sites are between 4 and 20 base-pairs apart. Detloff et al. (1992) also showed HR at the *HIS4* locus of yeast in a cross having alleles 26 bp apart. They used a palindromic insertion mutation as a PMS allele. The distance between alleles tested in this study ranged from 8 to 687 bases. The HR phenomenon was observed between alleles 8 and 20 bp apart and is not seen with longer distances between the markers. In this study, three types of alleles which commonly show PMS in yeast were used: (i) G to C transversion (*his1-672*); C to G transversion (*his1-216*) (ii) deletion (*his1-49*) and (iii) a palindromic insertion (*his1-876*). The HR phenomenon is seen with all three types of PMS alleles studied.

From the results of this study and observations at *CYC1* (Moore et al., 1988) and *HIS4* (Detloff et al., 1992), it can be generalized that the HR phenomenon can be observed in yeast in crosses involving a PMS allele and a normal allele as long as the distance between the two alleles is short (4-26 bp). Hyper-recombination at the *CYC1* locus was interpreted to be caused by differential repair of one allele over the other (Moore et al., 1988). As shown in FIG. 21, if a G to C mutant allele is repaired inefficiently and the other allele is repaired with normal efficiency, HR can be

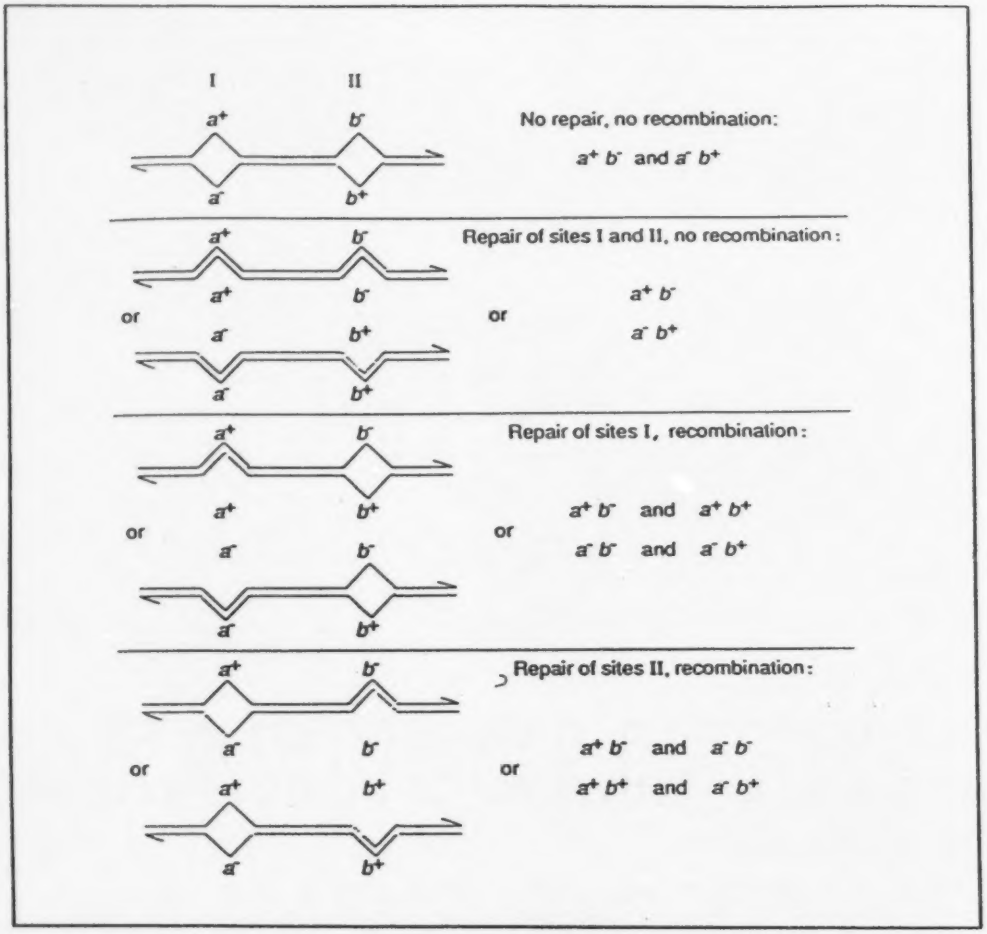

FIG. 21. Diagram illustrating the outcome of repair of two mismatched base-pairs at sites I and II of the heteroallelic cross $a^+b^- \times a^-b^+$.

observed. In yeast, alleles with G to C transversion mutations show postmeiotic segregation. It has been shown by Leblon and Rossignol (1973) in *Ascobolus* that when a high PMS allele is placed near a low PMS allele, the high PMS allele is co-converted and takes the recombination spectrum of the normal allele. As discussed in the Review of Literature this has also been observed in yeast. In a heteroallelic cross, the PMS allele is co-converted by the normal allele. Co-conversion will not yield any prototrophs (FIG. 21). Only differential repair can give prototrophs which is seen as hyper-recombination. Based on these observations we postulate that G to C mutations at the *CYC1* locus may be PMS alleles and the differential repair could be the result of the short distance between the PMS allele and the normal allele in those crosses. We propose that the differential repair is achieved by failure of mismatch repair at the PMS allele and correction of mismatch at the normal allele. We postulate that the prototrophs in the crosses showing hyper-recombination arise mostly from postmeiotic segregation.

2. Postmeiotic segregation produces hyper-recombinants

The presence of postmeiotic segregation in the hyper-recombination prototrophs was tested in the diploid PM130 (*his1-216 X his1-1*). In this cross both haploid strains were histidine auxotrophs. Prototrophs arise mainly by recombination and according to our hypothesis they should show PMS. The random spore and unselected tetrad analyses showed that prototrophs are associated with postmeiotic segregation as we predicted. These two results show directly that HR is due to postmeiotic segregation of the PMS allele and normal correction of the other allele. Other circumstantial evidence supporting our hypothesis is seen from the study of P1 and P2 classes of prototrophic recombinants (please refer to the results section for the details of P1 and P2 classes). As the polarity gradient of NMS (Non-Mendelian segregation) at the *HIS1* locus is from 3' to 5', the P1 class represents the conversion of the *his1* allele at the 3' (high conversion) end and P2 at the 5' side. Two-point crosses

having normal recombination frequency show P1>P2 (Table 15). The greater frequency of P1 over P2 is normal for any two-point cross. PM130, PM151 and PM152 have the PMS alleles at the high conversion end of the locus. In those crosses P2>P1 (Table 16). This shows that heteroduplex resolution in HR crosses may be different from the crosses showing normal level of recombination.

3. Postmeiotic segregation results from blockage of mismatch repair

The above correlation of PMS with hyper-recombination suggests that failure of repair of the PMS allele is responsible for two events: one is postmeiotic segregation at the PMS allele and the other is blockage of co-correction. We suggest that the PMS event itself results from blockage of mismatch repair after recognition rather than from non-recognition of mismatches. There is a great deal of evidence to support this postulate. In yeast, the mismatch repair system is very efficient and most types of base mismatches are repaired efficiently. As discussed in the Review of Literature, only three classes of alleles show PMS: (i) G to C transversion mutants (ii) some deletion mutants (*his1-49* and *ade8-18)*, and (iii) perfect palindromes.

In vivo and *in vitro* studies show that C/C mismatches are not corrected efficiently (Please see the review). So it was postulated that the C/C mismatch is not recognized and evades mismatch repair (Claverys and Lacks, 1986). However, DNA foot-printing analysis of the mismatch binding proteins (Su and Modrich, 1986) showed that C/C is also recognized, but the dissociation constant of C/C is very high compared to other mismatches. They suggested that repair efficiency of these lesions is inversely proportional to the dissociation constants of MutS protein from the mismatches. In disagreement, Su et al. (1988) have shown that A/C and C/C have similar dissociation constants. However, A/C mismatches are repaired very efficiently compared to C/C mismatches in *E. coli*. Thus, the efficiency of repair of mismatches is not dependent on the dissociation constant. In yeast, the repair system is comparable

to *E. coli* in that A/C mismatches are repaired more efficiently than C/C mismatches. These results imply that C/C is recognized but not repaired. Based on the hyper-recombination phenomenon, we propose that C/C is recognized by a mismatch repair protein but not repaired, and PMS results from a failure of repair to proceed past the recognition step.

In yeast, most heteroduplexes containing deletions are repaired very efficiently. White et al. (1988) found that deletion heteroalleles in the *ADE8* locus of yeast which show PMS created a consensus sequence in the flanking regions of the deletion similar to the sequences found in recombination hotspots. They proposed that the proteins involved in recombination may bind to this sequence and block the repair, resulting in PMS. In *S. pombe*, the *ade6-M26* mutation, a single base-pair change, creates a recombination hotspot (Ponticelli et al., 1988). A similar sequence is found in the deleted part of the *his1-49* sequence but in opposite polarity (FIG. 3). This also suggests that PMS of *his 1-49* in heteroallelic crosses may be due to binding of a protein. The protein which may bind to the loop in a *his1-49* and wild-type heteroduplex would be able to recognize the sequence in single-stranded DNA independently of chemical polarity. These results again suggest that postmeiotic segregation is due to blockage of mismatch repair.

Palindromic DNA sequences are common in both prokaryotes and eukaryotes. *In vitro*, it has been shown that they can form intra-strand base-pairs (Gellert et al., 1979) creating a secondary structure in the DNA but it is not clear whether this type of configuration is formed *in vivo*. Kinetic studies suggest that a cruciform is not likely to be common *in vivo* (Gellert et al., 1983). Electron microscopic examination of psoralen-cross-linked DNA failed to reveal cruciforms in bacterial and Drosophila DNA (Cech and Pardue, 1976; Sinden et al., 1983). However, there are several indications that DNA secondary structures may be biologically important. First, long palindromes in plasmid and bacteriophage DNA are unstable although these sequences

can be partly stabilized in nuclease-deficient strains of *E. coli* (Collins, 1981 and Chalker et al., 1988). Palindromes greater than 26 bp in length stimulate deletion formation (Weston-Hafer and Berg, 1991). Some mutations in *E. coli* appear to reflect mismatch correction occurring between palindromic repeats (Ripley and Glickman, 1983). Chang and Murialdo (1990) found that interaction of a nuclear protein with a palindromic sequence within the mouse immunoglobulin λ2-chain promoter is necessary for its transcription. McMurray et al. (1991) also showed that the human enkephalin gene enhancer has a perfect repeat, which has the potential to form a cruciform structure, and intact repeats are necessary for enhancement of that gene. The efficient recognition of promoters by N4 virion RNA polymerase requires single-stranded DNA with a hairpin structure at a specific position. Many cellular proteins recognize secondary structures in RNA. Hence it was postulated that some kind of protein is bound to the mismatch at the PMS allele which results in PMS. The above evidence combined with the results of this study suggest that postmeiotic segregation in yeast is not due to evasion by non-recognition, but due to the blockage of mismatch repair by binding of either a mismatch repair protein or of some other protein.

4. Molecular model

Based on the evidence reported herein, a molecular model is proposed to account for the hyper-recombination phenomenon. This model is based on the *E. coli* mismatch repair system. It is reasonable to suppose that mismatch repair in yeast may be analogous to *E. coli* since there are genes in yeast which have sequences homologous to *E. coli* mismatch repair genes. The *PMS1* gene has homology to the *MutL* gene of *E. coli*. A mutation in this gene causes a deficiency in mismatch repair and produces an increased level of PMS and a decreased level of gene conversion frequencies, even in normal alleles (well repaired alleles). Genes homologous to *mutS* have also been identified in yeast. Reenan and Kolodner (1992) reported two *mutS* homologues of

yeast. One of them is involved in mitochondrial mismatch repair and the other one is involved in mismatch repair in the nucleus. Four different *mutS* homologues have been identified in yeast. All of them have striking homology at their carboxy termini and this sequence is conserved from *E. coli* to mammals (personal communication from Dr. M. Radman). This suggests that there may be redundancy in *MutS* genes in yeast. The other possibility is that all the genes are necessary, each one acting at a different stage in the recognition and repair of mismatches or all may be involved in the formation of a complex that is in turn involved in the various steps of mismatch repair.

In *E. coli*, mismatches are recognized by MutS protein. The binding of MutS to the mismatch stimulates the binding of MutL to MutS (Modrich, 1989). The MutL and MutS proteins may open up the site of a mismatch allowing MutU protein (DNA helicase II) to enter the DNA and unwind it bidirectionally. The MutH protein introduces single-strand breaks, allowing the displacement of single-stranded DNA carrying the mismatched bases. In *E. coli*, MutH protein activity may be replaced by an existing nick during *in vitro* repair. The resulting gap is filled by DNA polymerase using the intact DNA strand as the template. The nick is then sealed by ligase (Langle-Rouault et al., 1987). If the homologous proteins in yeast behave in a similar manner, the mismatch may be recognized by the MutS homologues and this complex of MutS with the mismatch may be bound by the MutL homologue. A nick may be made by an endonuclease or existing nicks may be used. Mismatch-specific endonuclease activity has been seen in extracts from yeast (Chang and Lu, 1991). This endonuclease activity has been detected for all possible mismatches. Nicking activity is lowest for T/C, C/A and C/C mismatches. The nicks have been mapped at 3 places at the second, third and fourth phosphodiester bonds 5' to the mispaired base. Extracts from human cells also have mismatch specific nicking activity (Yeh et al., 1991). This enzyme system makes incision at the first phosphodiester bond 5' to the mispaired bases. Strand

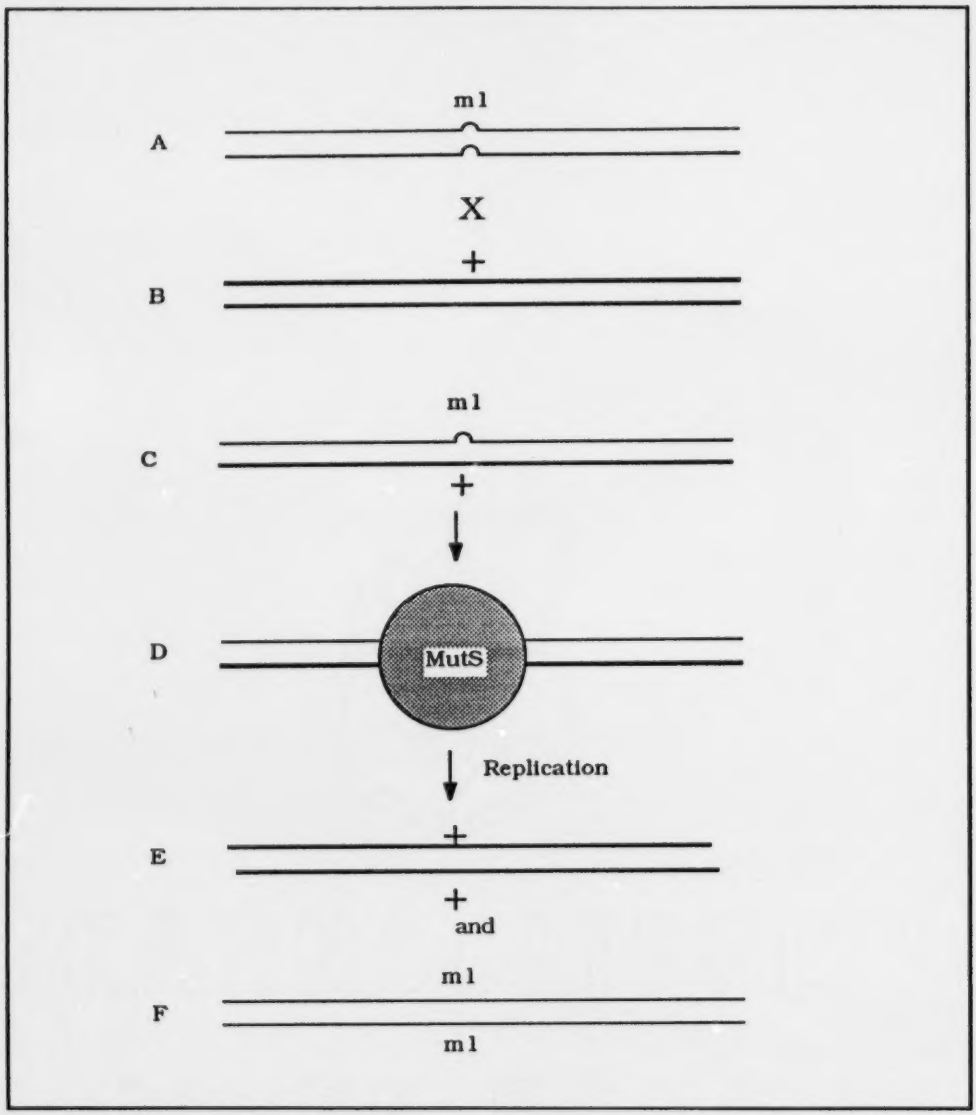

FIG. 22. Molecular model for postmeiotic segregation.

A cross between a PMS allele m1 and wild type + (A and B) may form a m1/+ mismatch (C) in the heteroduplex. The mismatch is recognized by MutS homologues or some other protein (D). An unrepaired mismatch segregates postmeiotically to + (E) and m1 (F). (Please see the text for details)

displacement may be due to the action of helicases. The displaced strand may be eroded by exonuclease or cut by an endonuclease. This leaves a single-strand gap which may filled by polymerase using the opposite strand as a template. In yeast, proteins which have endo-and exo-nuclease activity have been reported (reviewed by Resnick, 1987). Endo- and exo-nuclease activity may be present in the same protein or in different proteins. Genes having helicase activity have been reported in yeast. For example, the *RAD3* gene has been reported to be involved in excision repair and has helicase activity (reviewed by Friedberg, 1988). In the model proposed here, PMS alleles are recognized and bound either by a mismatch repair protein (MutS homologue) or by some other proteins. This repair does not proceed further, resulting in PMS after DNA replication (FIG. 22). PMS is scored as a sectored colony, the sectors showing two genotypes.

With a normal allele, repair is efficient and the outcome is either conversion or restoration. During repair, after recognition, a nick is made and strand displacement may take place bidirectionally by helicases, which may act separately or as a complex. As mentioned previously, the conversion tract, which may represent the repair tract in yeast, is long and is usually continuous (for details please refer to the "Review of Literature"). During mismatch repair there are three different possibilities depending on the proteins involved in the repair process. In the first possibility, if the repair has polarity, the repair may proceed either towards the 3' side or towards the 5' side exclusively and the repair will be unidirectional. The second possibility is that the repair may go unidirectionally with equal probability towards the 3' side or the 5' side. This type of unidirectional repair will form single-strand gaps from the mismatch to a certain distance either on the 3' side or on the 5' side. The third possibility is that the repair goes bidirectionally. In this type of repair the single-strand gaps may be formed on both sides of the base mismatch. In the heteroduplex formed at normal alleles of yeast, it seems that repair goes bidirectionally and a stretch of single-stranded DNA is stripped off from the normal allele on both sides of the mismatch. A single-strand gap

is formed and is filled in using the opposite strand of DNA as template. The bidirectionality of repair is implied by the following genetic data. The allele *his1-49* shows 85% PMS (Hastings, 1984) when alone, but PMS drops to 24% in the presence of another allele, *his1-1S*. If there were polarity or unidirectionality in mismatch repair, one would expect the repair from the normal allele (*his1-1S*) to go 50% to the right side, and 50% to the left, thus reducing PMS in *his1-49* only to 43%. The result of the cross involving *his1-49 x his1-1S* alleles suggests that, at the population level, there is no polarity involved in the repair. There are other data to support this interpretation. Detloff and Petes (1992) studied a heteroallelic cross between a PMS allele and a normal allele. They showed that the allele *his4-519* which shows no PMS and is located 26 bp from the allele *his4-IR9* (which shows 80% PMS) causes a five-fold decrease in the frequency of PMS at *his1-IR9* (from 28% to 6% of the total tetrads). This decrease is compensated for by an increase in the frequency of gene conversion of *his4-IR9* from 8% to 26%. Almost all conversions (98%) at the *his4-IR9* allele were co-conversions and 85% of conversions at *his4-519* include the allele *his4-IR9*. The results of this study also suggest indirectly that there is no polarity in mismatch repair. The hyper-recombination phenomenon is seen on both sides of PMS alleles. In this study, the crosses with the PMS allele *his1-672* and normal alleles on either side (*his1-30,1-40* and *1-51*) showed hyper-recombination. *In vitro* studies with crude extracts of *Xenopus* eggs showed that mismatch-repair provoked nucleotide incorporation is observed at distances of 0.5 kb on either side of the mismatch. The most pronounced region of increased synthesis is 150 bp on either side (Brooks et al., 1989). Gap formation associated with methyl-directed mismatch correction under conditions of restricted DNA synthesis was studied by Su et al. (1988). Their results indicate that a single-strand gap is formed in the regions of the DNA molecule containing the mismatches and the d(GATC) site. They found that the gap extends on both sides of the mismatches in a single DNA molecule and often doesnot include the d(GATC) site. These studies

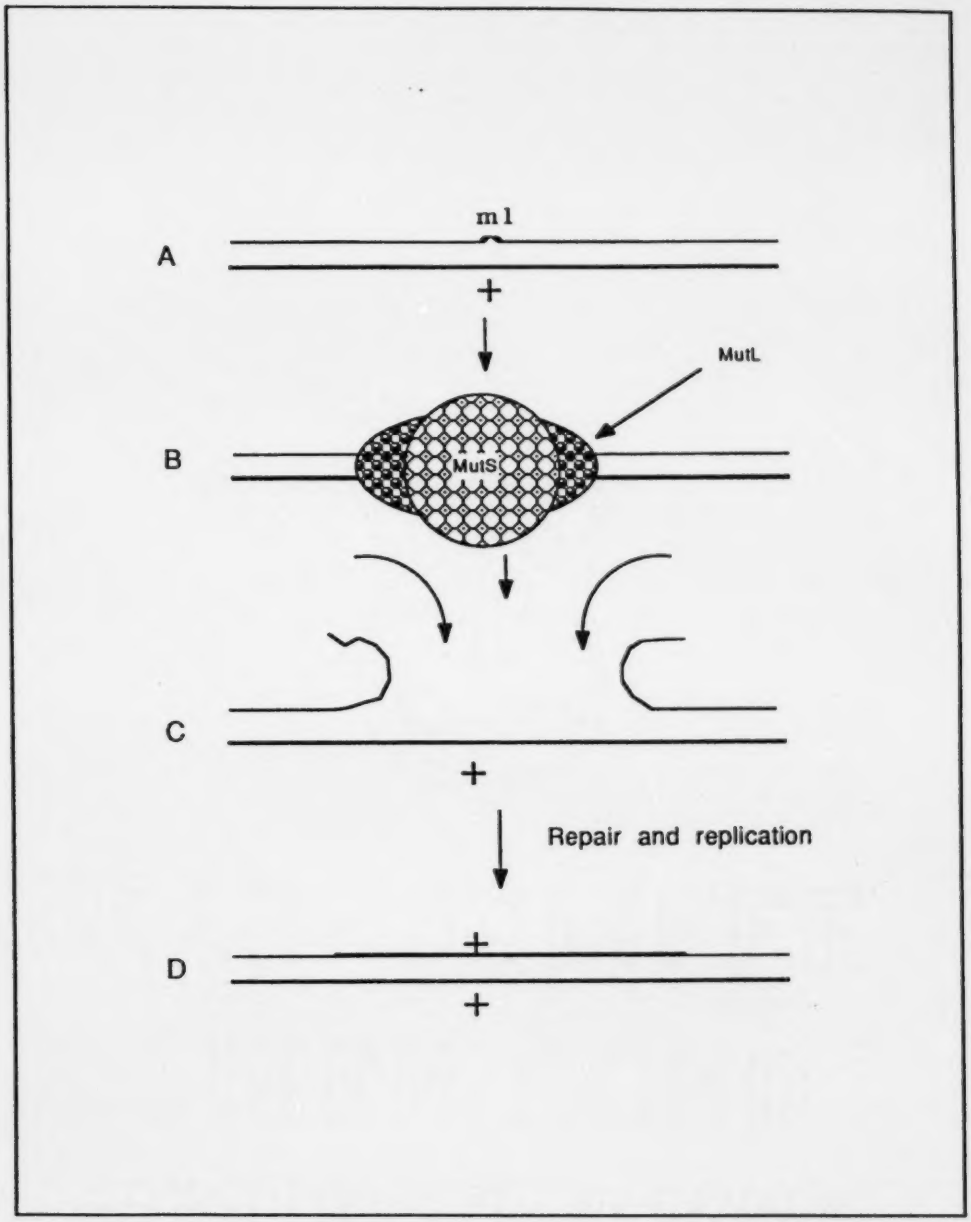

FIG. 23. Molecular model for repair at mismatches formed at normal alleles.

A mismatch m1/+ (A) is formed in a cross between a mutant (m1) and wild type (+). The mismatch is recognized by mismatch repair proteins (B) which start repair by creating a nick and displacing one DNA strand bidirectionally (C). The displaced DNA strand may be eroded, resulting in a single-strand gap. The gap is subsequently filled using the opposite strand as a template (D). (Please see the text for details).

show that during co-repair, the repair events may go in either or both directions and may end at different distances equally on both sides of the mismatch. The mechanism that controls the length of the repair patch is not known. During excision, single-stranded DNA may be excised after displacement or without displacement by the concerted action of endo-and exo-nuclease activity. Neither possibility is ruled out.

A molecular model for the repair of normal alleles is shown in FIG. 23. In a cross between a mutant (m1) and wild type (+), the heteroduplex will include both of the alleles (m1/+). After formation, the heteroduplex is recognized by the mismatch repair proteins and one of the two strands is excised as discussed earlier. The gap formed by this process is filled by polymerase followed by ligation of the remaining nick. If the strand with the m1 allele is excised (panel C of the FIG. 23), the resulting phenotype is a prototroph (+) (panel D of the FIG 23). If the other strand is excised the resulting phenotype is auxotrophic (m1). During repair the two strands may not be excised equally. There are preferences and discriminations in excision of DNA strands during repair (for details please refer to the "Review of Literature")

A. Co-correction of a PMS allele with a normal allele

We propose that when a PMS allele is located at some distance from the normal allele, the repair that starts at the normal allele displaces the protein bound at the PMS allele probably by helicase activity (FIG. 24). As the repair tract is continuous and long, the PMS allele is co-corrected. In yeast, the average length of conversion tracts varies from 0.5 kb to 1.5 kb. If the distance between the normal allele and the PMS allele is within 0.5 kb it is likely that the PMS allele will be included in the correction tract and co-corrected. In other words, failure to repair the PMS allele does not affect the probability of co-correction. During co-correction there is no polarity, that is, the PMS allele is co-corrected whether it is located on the left (5' side) or on the right (3') of the normal allele, due to bidirectionality of repair. The allele *arg4-16* is a PMS allele and

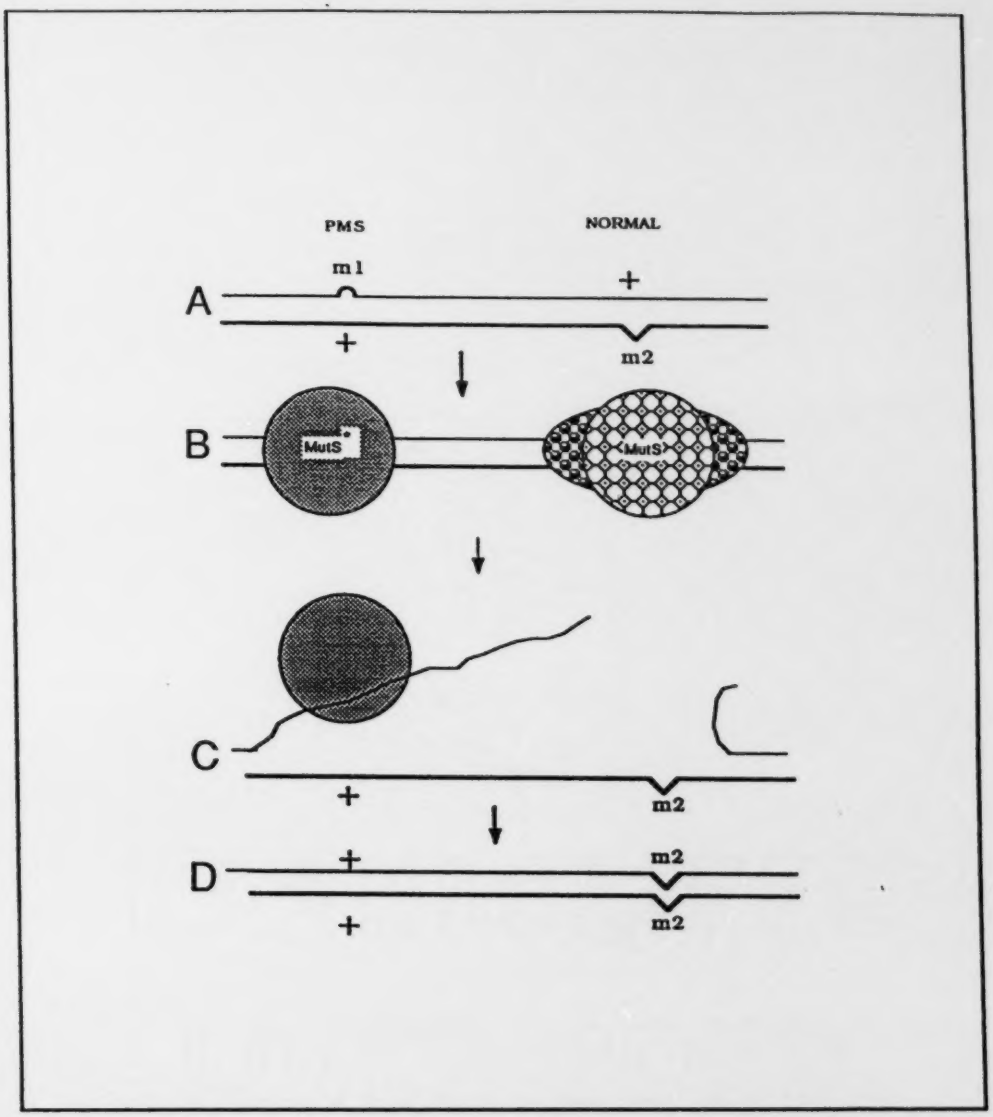

FIG. 24. Molecular model for co-correction of a PMS allele by a normal allele.

In a cross between a PMS allele (m1) and a normal allele (m2) mismatches may be formed at two alleles simultaneously (A). Both mismatches may be recognized by mismatch repair proteins (B). Mismatch repair at the PMS allele does not proceed beyond the recognition step (MutS*). Repair at the normal allele may proceed bidirectionally and displaces the protein(s) bound at the PMS allele (C). The single strand gap which is formed by the excision of the displaced strand is filled by using the opposite strand as a template (D).

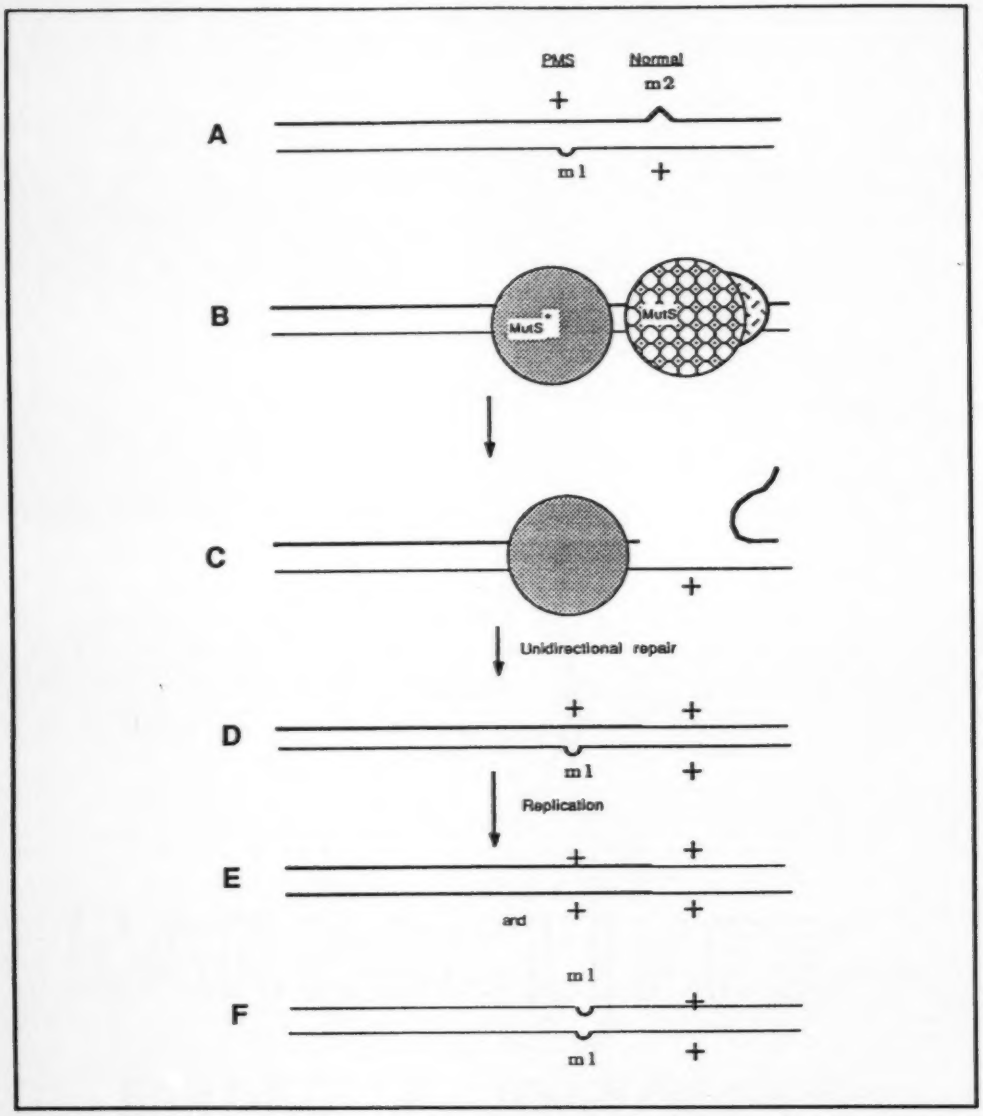

FIG. 25. Molecular model for hyper-recombination observed in crosses between a PMS allele and a normal allele.

In a cross between a PMS allele (m1) and a normal allele (m2) mismatches may be formed at the two alleles simultaneously (A). Both mismatches may be recognized by mismatch recognition proteins (B). Mismatch repair at the PMS allele does not proceed beyond the recognition step. This binding at the PMS allele restricts repair originating from the normal allele to one direction (C). This results in postmeiotic segregation at the PMS allele and correction at the normal allele (D). After DNA replication, the resulting genotypes are either ++ (E) and m1+(F).

PMS decreases when normal alleles are placed on either side (5' or 3') of this allele (Fogel et al., 1979). Co-correction will not yield HR since the resulting phenotype from co-correction is auxotrophic for the markers (m1+ or +m2) involved in the cross (FIG. 24).

B. Hyper-recombination occurs when there is a short distance between a PMS allele and a normal allele

A cross between a PMS allele and a normal allele will show hyper-recombination if the distance between the two alleles is between 4 and 26 base pairs. We propose that the mismatches at the PMS allele are bound by a protein, and excision does not proceed further. Mismatch repair protein binding is also not formed into a bidirectional repair complex on the normal allele because of the proximity of the protein binding to the PMS allele heterodplex (FIG. 25).

The protein(s) bound at the mismatches of the PMS alleles may differ depending on the type of mismatch. Three types of PMS alleles were tested in this study. Each one may be acting differently in the formation of PMS. In G to C transversion alleles, G/G or C/C mismatches are formed. If the G/G mismatch is repaired well, only a C/C mismatch can give PMS. We propose that C/C is recognized and bound by MutS homologues but not repaired. Though it may be recognized, the C/C mismatch may not make the right conformational change in MutS to be recognized by other proteins, like MutL, to complete the repair process. Another possibility is that C/C may allow the formation of the whole complex but may not allow repair to proceed due to structural distortion in the DNA. It is likely that only the MutS homologue is bound. Grilley et al. (1989) suggested that the MutL protein exists as a dimer. DNaseI protection experiments demonstrate that purified MutL protein interacts with a MutS-heteroduplex DNA complex in the presence of ATP. MutL protein does not interact with mismatches. DNaseI foot-printing analysis showed that the 20 bp protected by MutS protein

increased to 143 bp when MutL is present with MutS. The protection was seen on both sides (Su and Modrich, 1986). If MutL were to bind to MutS at C/C mismatches the complex would cover other mismatches in the normal alleles in that short distance. If both alleles were covered, differential repair and hyper-recombination could not happen. In the deletion mutation, the proteins involved in the recombination hotspot may be bound and block repair. With palindromic insertions, a protein that recognizes the hairpin may be involved in the blockage of repair. The possibility of binding of MutS homologues in the deletion/insertion PMS alleles is not ruled out.

5. Extension of the molecular model

The extent of protection will depend on the type of protein bound at the mismatches. The variations are shown in FIG. 26. If the protein(s) are bound symmetrically, protection is the same on both sides. If the normal allele is very close we can expect co-PMS or co-correction. Hyper-recombination is observed only beyond a certain distance. In *cyc1* mutants, hyper-recombination is not seen if the distance is 2 bp (Moore et al., 1988). If there is a competition and if the normal allele is recognized first it will lead to co-correction. Hyper-recombination is obtained only if the PMS allele is located within a certain distance. Su and Modrich (1986) have shown that the *E. coli* MutS protein protects 8-12 bp around the mismatch. In the case of a G/T mismatch it was 22 bp. The protection may not be symmetrical. The asymmetry depends on the type of mismatch. For example G/A and T/C mismatches at the same position showed opposite asymmetry in foot-printing. Thus depending on the type of mismatch, different consequences may be seen as shown in FIG. 26.

If the PMS allele is recognized first, and the protein(s) bound to the PMS allele covers the normal allele, both will be protected resulting in co-PMS. If only the PMS allele is protected leaving the normal allele available for correction, unidirectionality of repair will result in hyper-recombination. If the normal allele is recognized first

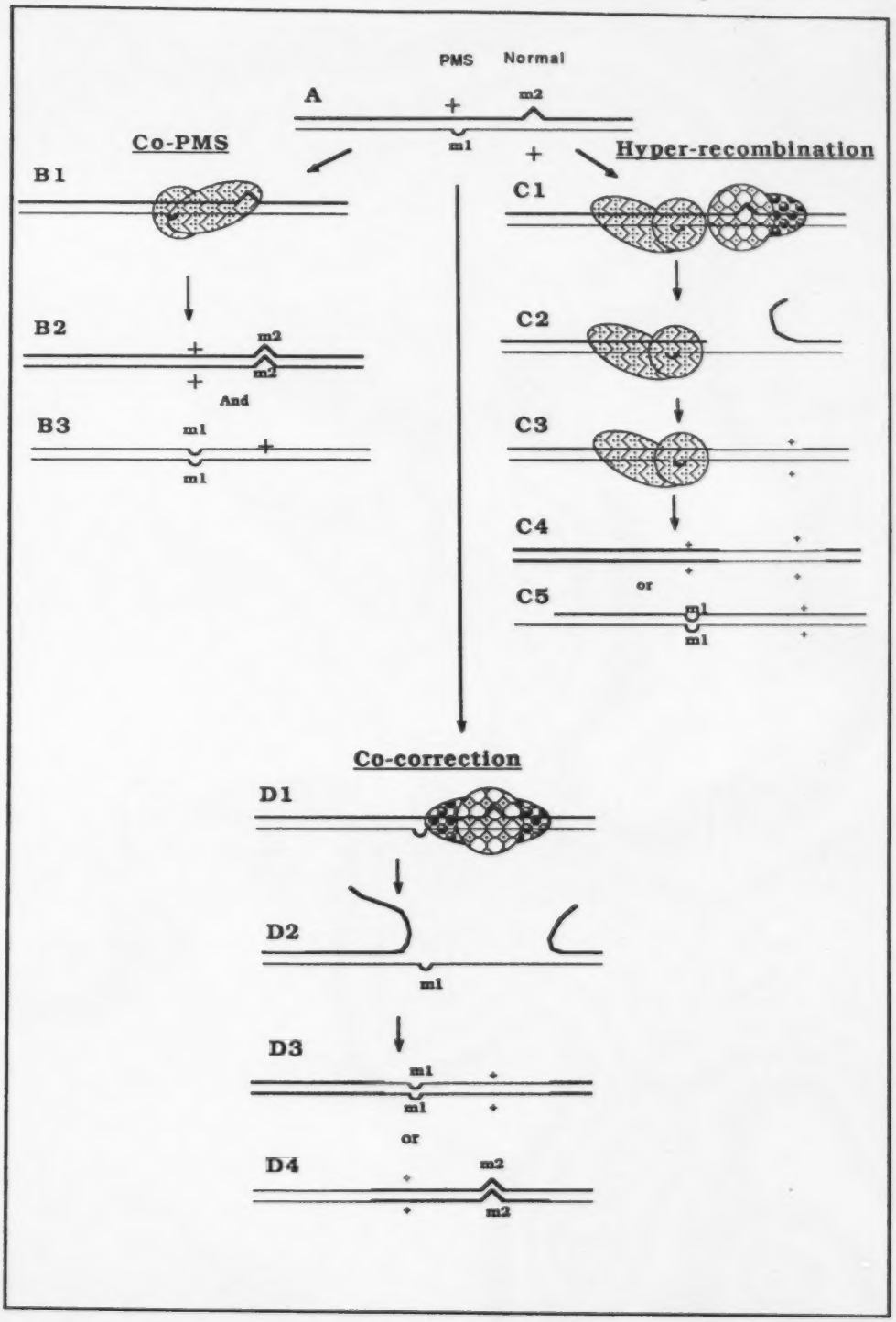
PMS
Normal
A
m2
m1
Co-PMS
Hyper-recombination
B1
C1
B2
m2
m2
And
C2
B3
m1
m1
C3
C4
or
C5
m1
m1
Co-correction
D1
D2
m1
D3
m1
m1
or
D4
m2
m2

FIG. 26. Extension of the molecular model for hyper-recombination.

In a cross between a PMS allele (m1) and a normal allele (m2), mismatches may be formed at the two alleles simultaneously (A). If binding of the mismatch repair protein complex at the PMS allele is asymmetrical this protein may cover the normal allele on only one side (B1). This results in co-PMS due to blockage of repair at both alleles (B2 and B3). If the proteins bound at the PMS allele extends on the other side (C1) this may leave the normal allele free to repair unidirectionally (C2). This results in HR (C3 to C5). If the mismatch at the normal allele is recognized first (D1) this may result in co-correction of both alleles (D2 to D4).

and the repair starts before the PMS allele is recognized, both alleles will be co-corrected. With the same mismatch any of the three events can occur. Detloff and Petes (1992) studied a diploid constructed from a PMS allele (palindromic insertions) and a normal allele at a distance of 26 bp within the *HIS4* locus. They showed segregation patterns representing all three events from the same diploid: (i) co-PMS, (ii) PMS (5:3) at the PMS allele and normal conversion (6:2) at the non-PMS allele and (iii) co-conversion of both alleles.

6. Repair in heteroduplexes having two PMS alleles

A cross between two PMS alleles may show two different types of outcome depending on the PMS alleles used. For example, a palindromic insertion or a deletion like the *his1-49* mutation will form a loop in the heteroduplex formed with the wild-type allele. Both types of heteroduplex may be protected. On the other hand, if the PMS allele is a G to C transversion mutation two type of mismatches (G/G or C/C) are possible. Many studies have shown that G/G mismatches are repaired very well both *in vivo* and *in vitro* but C/C mismatches are not. In yeast, it has been shown that C/C mismatches show PMS (for details please refer to the Review of Literature). As the G/G mismatches are repaired very well, only C/C mismatches can yield PMS.

If the cross is made with two insertion/deletion PMS mutant alleles, repair at both alleles is impaired. This will yield a very low level of recombinants. Theoretically this type of cross should yield no recombination unless there are heteroduplex endings between the two alleles. None of the PMS alleles show 100% PMS. Depending on the efficiency of repair in the PMS alleles the recombination frequency may vary. A cross involving one insertion allele and one G to C transversion allele may yield hyper-recombination if the alleles are within the short distance. As shown in the FIG. 27, a C/C mismatch is formed at one PMS allele (G to C), and a loop-out at the other

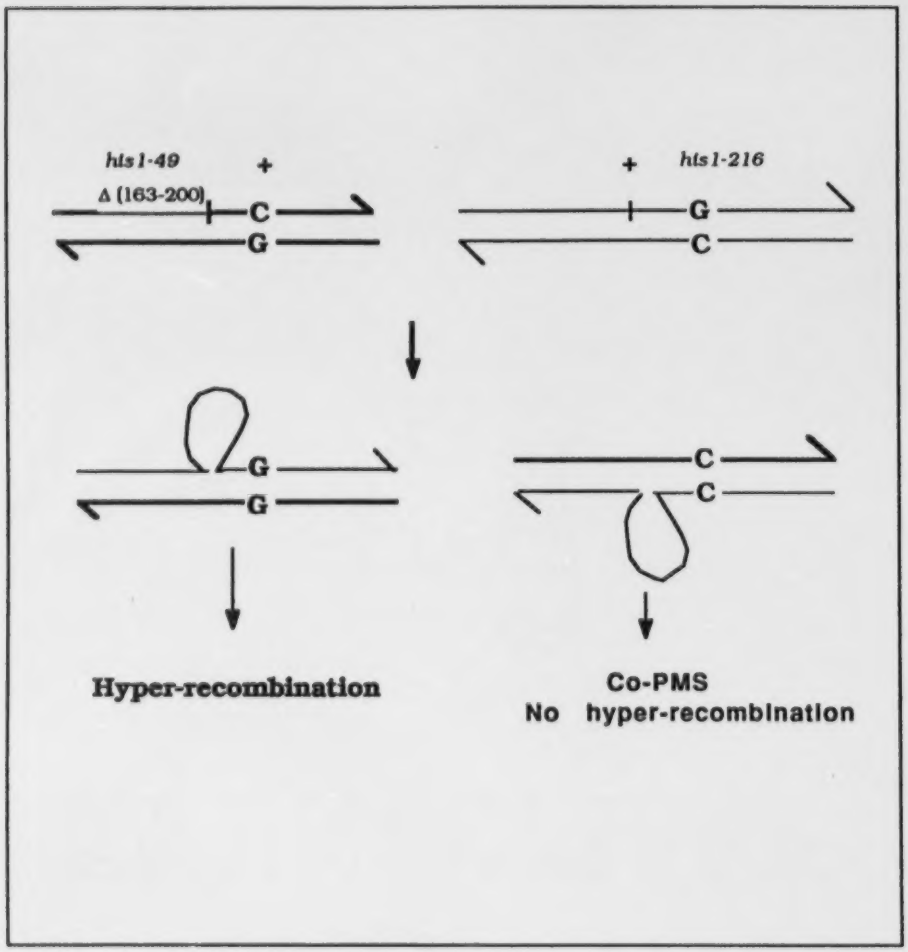

FIG. 27. Heteroduplex formation and types of mismatches in a cross between two PMS alleles *(his1-49 x his1-216)* (see the text for details).

allele containing the insertion or deletion; repair of both mismatches is inefficient and no differential repair is expected according to the model outlined above. However, if a G/G mismatch is formed at one PMS allele and a loop out occurs at the other allele, differential repair can be expected over a certain distance (4 to 26bp). This was tested in diploid PM131, made by crossing two haploids containing different PMS alleles (*his1-49* and *his1-216*). During heteroduplex formation in this diploid, the wild-type sequence covering the deleted part of *his1-49* will always loop out whether the coding strand or the non-coding strand is invading. The repair of this mismatch is blocked most of the time. At *his1-216*, either a C/C or G/G mismatch may be formed in the heteroduplex. If a G/G mismatch is formed at *his1-216* this will act like a normal allele because this lesion is repaired efficiently. This results in unidirectional repair at *his1-216* (FIG. 27). The unidirectionality of repair will produce hyper-recombination. HR is seen in this cross as predicted (Table 10). Recombination in PM131 (PMS x PMS allele) is comparable to recombination in PM120 (PMS x normal allele) and PM130 (PMS x normal allele). Hyper-recombination is seen in all these crosses, consistent with our model.

7. Repair in crosses involving two normal alleles

In a cross between two normal alleles, our model predicts that both alleles are repaired well and the genetic consequences of repair depend upon which allele is recognized first. Often the repair of one allele co-corrects the other allele. In this type of cross no hyper-recombination is expected since there will not be any differential repair. In this study, crosses between normal alleles showed variation in recombination frequency but none of them showed HR.

These results strongly support the proposal that postmeiotic segregation at PMS alleles and unidirectional repair at well repaired alleles produces HR (FIG. 25). The results presented in this study also support the assumption that heteroduplexes are

formed as intermediates in recombination since this type of result cannot be explained by the double-strand gap repair model although the double-strand gap repair model of Szostak et al. (1983) does include heteroduplex DNA at sites flanking the gaps. Savage and Hastings (1981) showed that heteroduplexes formed at the *HIS1* locus are continuous. A heteroduplex formed at the high conversion end of the gene appears to be extended into the gene and ends at various positions within it.

Another explanation for HR could be that a heteroduplex may end between two mismatches, and repair occurs in both directions from the allele which has a mismatch in the heteroduplex and this gives hyper-recombination. We consider that this is unlikely since the distance between alleles in crosses showing HR is so small that the chance of a heteroduplex ending between the two alleles should be probably rare. Also, if HR is due to termination of a heteroduplex between two specific alleles it should be seen in crosses involving two normal alleles. None of the crosses involving two normal alleles showed HR. No one has yet shown that PMS alleles enhance heteroduplex endings.

8. Lack of a polarity gradient in hyper-recombinants

Sequencing of several *his1* mutants, together with recombination data, suggest that the polarity gradient of gene conversion at the *HIS1* locus is in the 3' to 5' direction. This is opposite to the gradient found at *ARG4* or *HIS4*. The results of two-point crosses in this study confirm the direction of the polarity gradient (from 3' to 5') at the *HIS1* locus. The NMS (non-Mendelian) frequency for single-point crosses ranges from 3% to 1% from 3' to 5'. Based on this gradient, the HR frequency is expected to be at least three times higher for the crosses with *his1-876* than for other crosses showing HR, since *his1-876* is located at the high conversion end. However, there was not much difference in recombination frequency between the crosses. For example, crosses involving *his1-876* and *his1-49* showed almost the same frequency of recombination. These alleles are

located at opposite sides of the gradient. One of the reasons for this lack of a gradient may be that, in these crosses, three different PMS alleles are involved and they may be protected differentially by three different proteins, and the differences in protection may abolish the gradient. Another possibility could be that, during unidirectional repair, the PMS alleles may allow the other allele to repair in the same way (conversion or restoration type of repair) in all the crosses. Detloff et al. (1992) proposed that a gene conversion gradient reflects the direction of repair rather than the frequency of heteroduplex formation. They suggested that at the 5' end of *HIS4*, mismatch repair is mainly of the conversion type and at the 3' end it is of the restoration type. If that explanation is extrapolated to the *HIS1* locus, repair at the 3' end would be of the conversion type and at the 5' end it would be of restoration type, since the polarity gradient at this locus is from 3' to 5'. Since both conversion and restoration may contribute to HR, the polarity gradient would not be seen.

9. Unidirectionality of repair in hyper-recombinants

In the model we have proposed, repair of normal alleles is bidirectional. However, when a normal allele is in close proximity to a PMS allele (between 4 and 26 bp), repair is restricted to a single direction. This assumption of unidirectionality in repair in the HR crosses was tested in three-point crosses involving two PMS alleles and a normal allele. Two types of crosses were made. In the first cross, the normal allele was placed on one side (the high conversion side) of the two PMS alleles and in another cross the normal allele was placed between the two PMS alleles. The distance between one PMS allele and the normal allele is within the distance showing HR in the previous crosses. The experimental strategy is shown in FIG. 20. The diploids PM200 and PM201 have two PMS alleles in *trans* and a normal allele *his-40* or *his1-30*, respectively. As shown in FIG. 28 and 29, during recombination a heteroduplex,

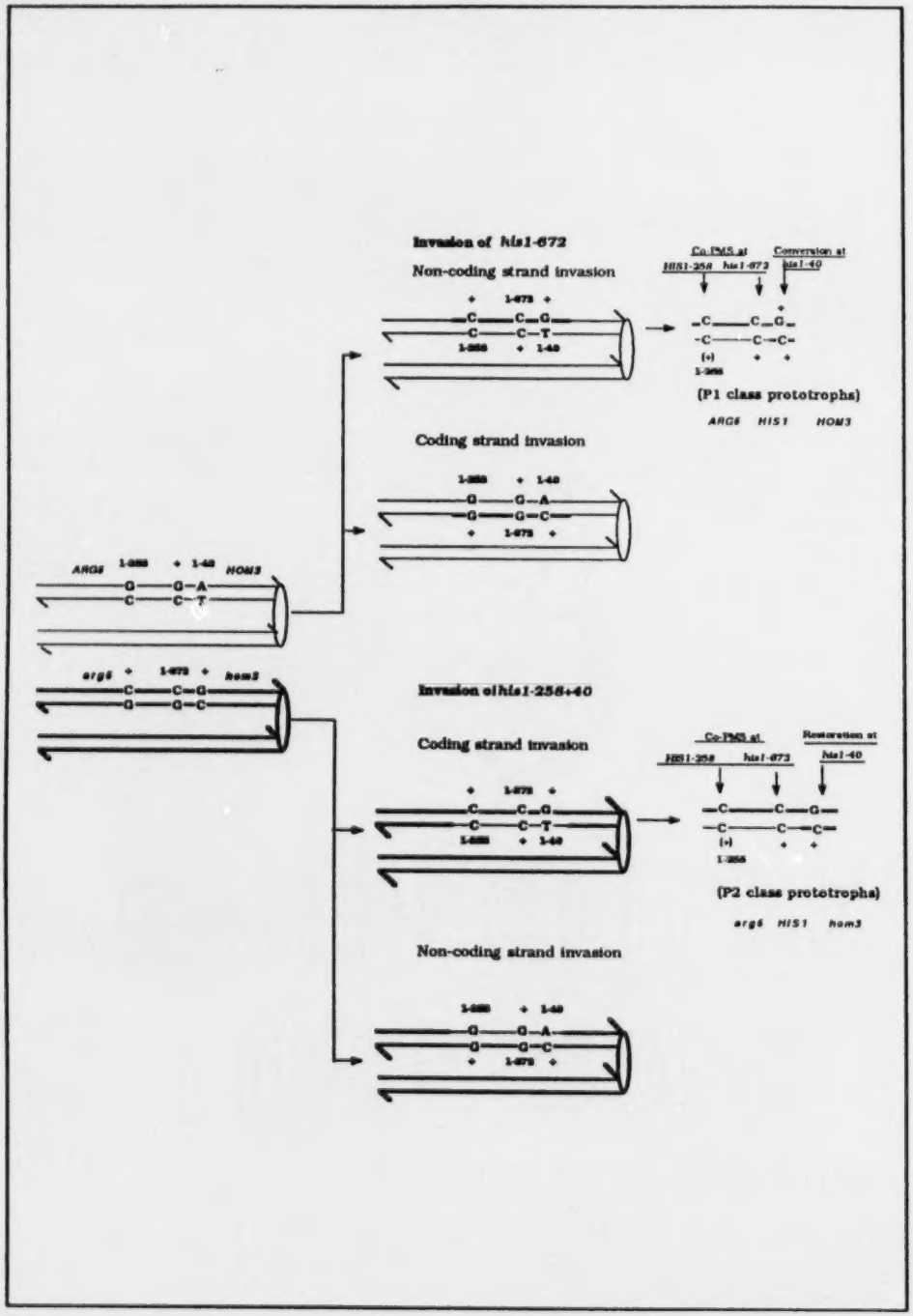

FIG. 28. Origin of P1 and P2 class prototrophs in the three-point cross *his1-672 x his1-258, 40* (PM200). (see the text for details).

covering all three alleles, would form either a G/G or a C/C mismatch at both PMS alleles.

In diploids PM200 and PM201 a G/G mismatch formed at both PMS alleles (FIG. 28 and 29) will result in the formation of an A/C mismatch at the normal allele (*his1-40* or *his1-30*). In this situation, whichever allele is recognized first, repair that starts from that allele may co-correct the other two alleles and no prototrophs would be expected. However, if a C/C mismatch occurs at both PMS alleles, (*HIS1-258* and *his1-672*) a G/T mismatch would be formed at the normal allele (*his1-40* and *his1-30*). If the G/T mismatch is recognized first, mismatch repair may co-correct the other two alleles and no prototrophs would be recovered. If the C/C mismatch is recognized first, binding of the MutS homologue protein may prevent the repair of the lesion at *his1-672*, as discussed earlier. I suppose that binding of the yeast MutS homologue protein at *his1-672* would also restrict mismatch repair from proceeding bidirectionally from the normal alleles (*his1-40* and *his1-30*). The C/C mismatch at *HIS1-258* is not repaired efficiently and would therefore not reduce the production of prototrophs by co-correction. As expected, these crosses showed hyper-recombination. During mismatch repair it is expected that, in the normal situation, the allele *HIS1-258* will be co-repaired with *his1-40* or *his1-30*. We suppose that the extension of mismatch repair from *his1-40* is stopped on one side by *his1-672* in the diploid PM200 and that this blockage protects the mismatch at *HIS1-258* from co-correction. However, in the diploid PM201, the normal allele is on the low conversion side of the PMS allele, *his1-672*. As there should be no blockage to the left of *his1-30* it is expected that the mismatch repair starting from *his1-30* co-corrects the other allele *HIS1-258* (FIG. 20). This postulate can be tested in the prototrophs from diploids PM200 and PM201 having parental marker configurations.

In diploid PM200, the P1 class represents prototrophs having the flanking marker configuration of the haploid parent with the *his1-40+HIS1-258* allele, and the

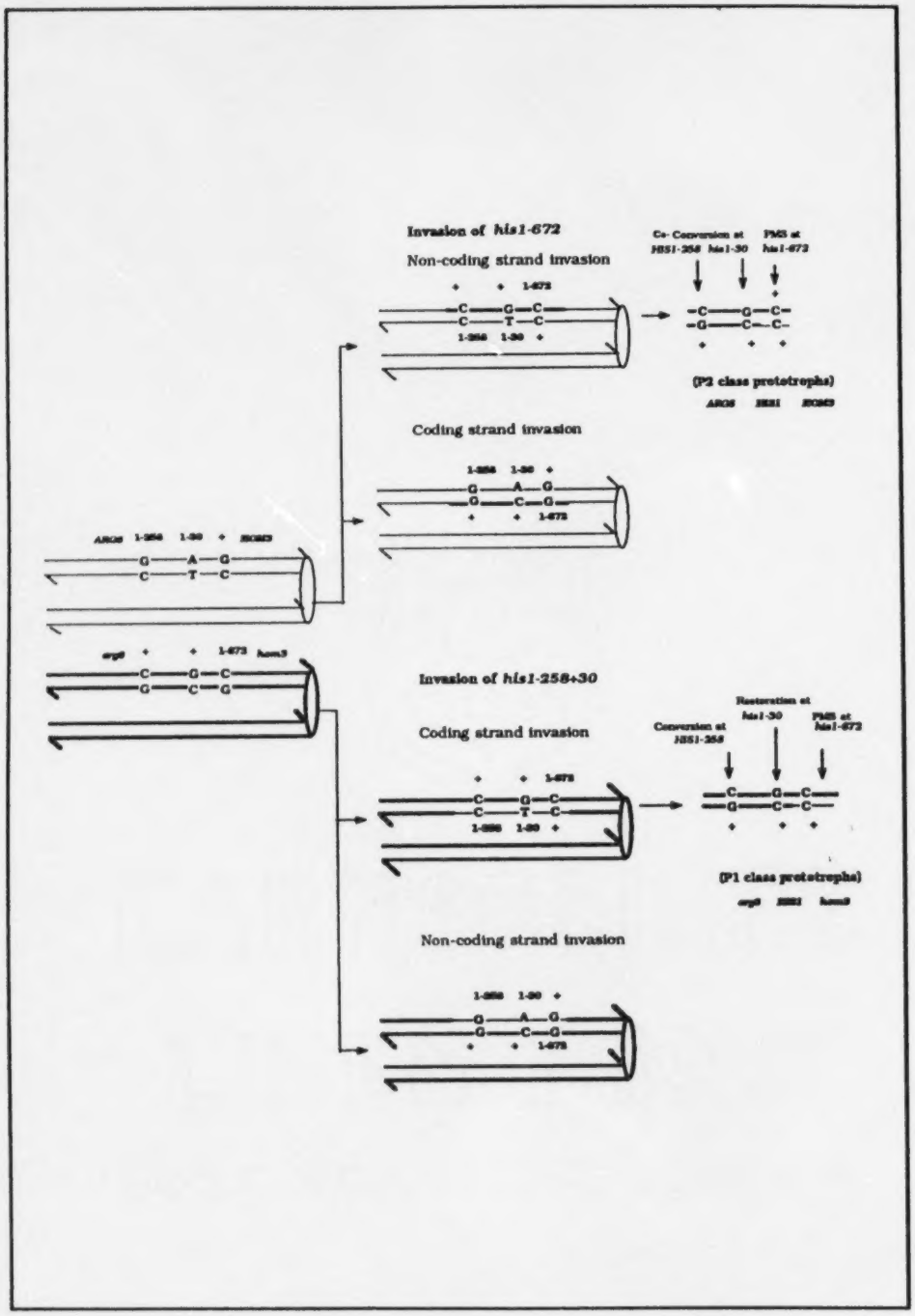

FIG. 29. Origin of P1 and P2 class prototrophs in the three-point cross *his1-672 x his1-258, 30* (PM201). (see the text for details).

P2 class represents those having the markers of the haploid parent with the *his1-672*. The P1 class of prototrophs is expected to arise by invasion of the non-coding strand from the chromatid having *his1-672* to the chromatid having *HIS1-258* and *his1-40* allele (FIG. 28). Conversion of *his1-40* and PMS at *his1-672* produces prototrophs of the P1 type only. Restoration of *his1-40* will result in auxotrophy. The P2 class of prototrophs is expected to arise by invasion of the coding strand from the chromatid having *HIS1-258* and *his1-40*, and restoration of the wild-type sequence at *his1-40* with PMS of *his1-672* on the chromatid having the *his1-672* sequence (FIG. 28). As the *HIS1-258* allele has no phenotype, co-correction or postmeiotic segregation of mismatches at this allele does not affect the recovery of P1 and P2 class prototrophs. P1 and P2 class prototrophs were analyzed by PCR amplification and restriction with *Sal*I. As the *HIS1-258* allele was made by elimination of a *Sal*I site from the wild-type, correction of the mutant allele *HIS1-258* to wild-type reintroduces the *Sal*I restriction site. None of the P1 type clones (0/14) showed the presence of a *Sal*I restriction site. In the P2 prototrophs, most of them (10/12) did not show co-correction with *his1-40* as expected.

In diploid PM201, the P2 class of prototrophs is thought to arise from invasion of the non-coding strand and conversion of the normal allele (*his1-30*). The P1 class of prototrophs would arise from the formation of heteroduplexes by invasion of the coding strand on the chromatid having the *his1-672* allele with restoration of wild-type at *his1-30* and PMS at *his1-672* (FIG. 29). As the *his1-30* allele is located on the low conversion side (left) of the PMS allele (*his1-672*), there will not be any blockage in progression of the repair tract which may extend up to the mismatch at *HIS1-258*. If the conversion tract which starts at *his1-30* also covers *HIS1-258*, co-correction will result in conversion of the *HIS1-258* allele to the wild-type. Co-correction of *HIS1-258* by *his1-30* can be detected by the presence of the *Sal*I restriction site. P2 class prototrophs were analyzed physically by PCR amplification of the genomic DNA isolated from these

prototrophs. When 13 of them were tested, seven showed the *SalI* site, thereby indicating co-correction. Molecular characterization of the P1 type of prototrophs showed that 9 out of 14 had the *SalI* site and were, therefore, co-corrected with *his1-30*.

The difference between the diploid constructs PM200 and PM201 is the location of the normal allele. In PM200, *his1-40* (the normal allele) is located on the right (high conversion) side of the *his1-672* allele and this cross shows no co-correction of the *HIS1-258* allele by the normal allele. However, in PM201 the normal allele (*his1-30*) is located on the left (low conversion) side of the PMS allele (*his1-672*) and shows co-correction of *HIS1-258* by the normal allele. These two results suggest that one PMS allele (*his1-672*) protects the other PMS allele (*HIS1-258*) and restricts repair which normally proceeds bidirectionally from the well-repaired allele (*his1-40*). This result supports a key prediction of the model described above.

In diploid PM200, none of the prototrophs is expected to show co-correction. However, one of the P2 class prototrophs shows the *SalI* site at *HIS1-258*. The reason for the presence of the wild-type allele at this site may be termination of the heteroduplex between the *his1-672 and HIS1-258* alleles. If the heteroduplex is not formed at *HIS1-258* the resulting prototrophs from restoration of *his1-40* will have only the *HIS1-258* sequence. In diploid PM201, it is expected that all the prototrophs will show correction of the *HIS1-258* allele to wild-type. However, this is not the case. As in diploid PM200, the reason for this could be ending of the heteroduplex between the *his1-30* and *HIS1-258* alleles, so that heteroduplex correction at the *his1-30* allele may not affect the *HIS1-258* allele. In diploid PM201, co-correction of *HIS1-258* shows that the heteroduplex frequently extends to *HIS1-258*. However, another possibility is that many repair tracts have a length of less than 400 bp from the marker being corrected so that the C/C mismatch at *HIS1-258* may not be corrected, resulting in PMS. This would be consistent with the studies of repair synthesis in *Xenopus* cited above (Brooks et al., 1989).

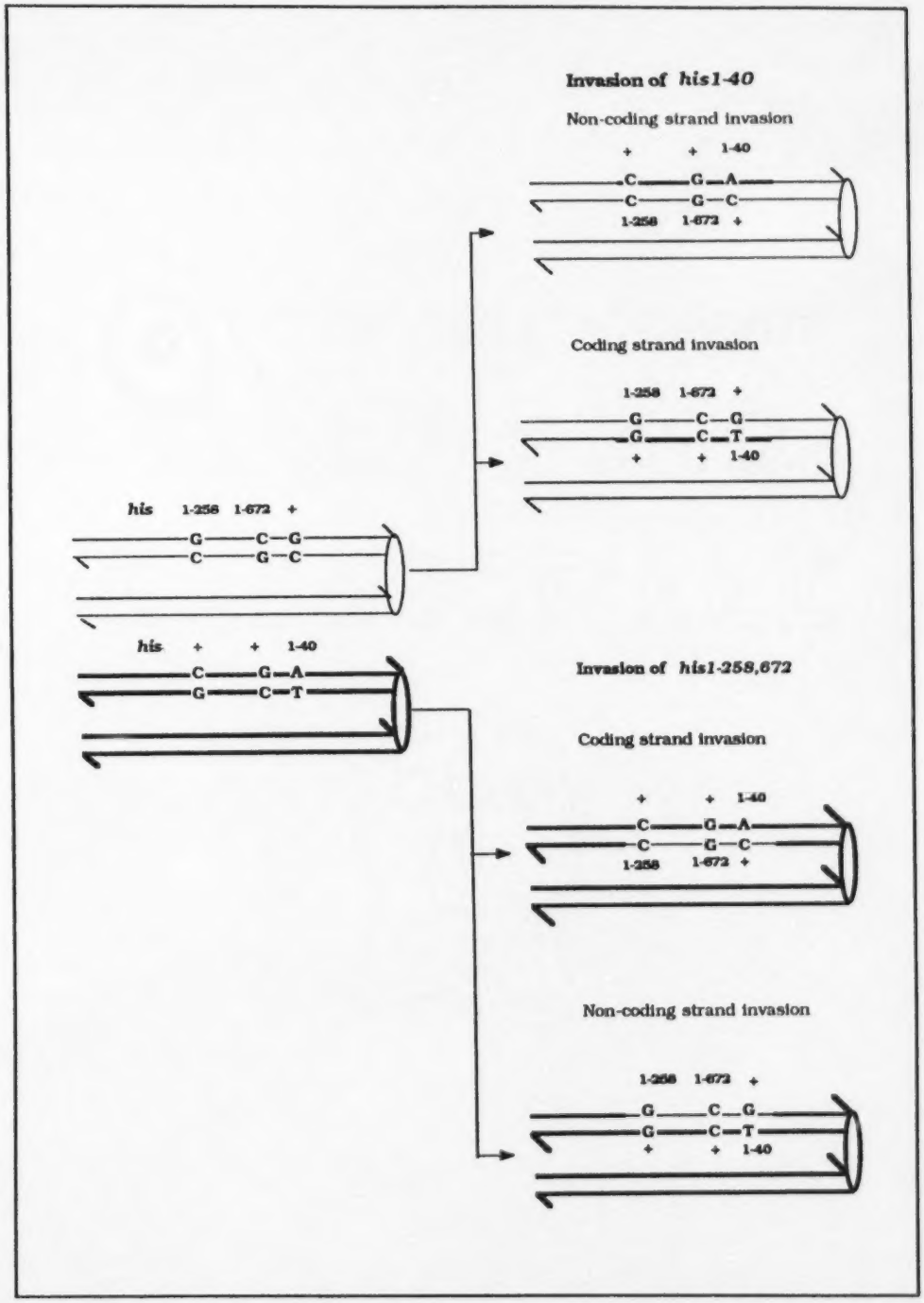

FIG. 30. Heteroduplex formation and types of mismatches in the three-point cross *his1-258, 672* x *his1-40* (PM300) (see the text for details).

The relative proportion of the parental classes of prototrophs depends on the proportion of coding and non-coding strand invasion and the frequency of correction by conversion or by restoration. Among the parental type of prototrophs, P1 class prototrophs occur more frequently than than those of the P2 class in diploid PM200. In diploid PM201, P2 class prototrophs are more frequent than the P1 class. As seen earlier, the P1 class in diploid PM200 and the P2 class in diploid PM201 arise by invasion of the non-coding strand and the conversion type of mismatch repair.

In another set of three-point crosses, the PMS alleles (*HIS1-258* and *his1-672)* were placed in *cis* so that if one PMS allele forms a G/G mismatch the other one will form a C/C mismatch (FIG. 30 and 31). In those crosses, the effect of a G/G mismatch on differential repair can be seen. A haploid strain with the double mutation (*his1-258,672*) was made in two steps (please see the Results section for details) and crossed to haploids having the *his1-40* or *his1-30* mutations to make diploids PM300 and PM301. In these diploids (PM300 and PM301), if a C/C mismatch is formed at allele *HIS1-258*, a G/G mismatch will occur at the other PMS allele *his1-672* and a G/T at the normal allele (*his 1-40* or *his1-30*) (FIG. 30 and 31). In this situation, recognition and repair from the mismatched G/G or G/T may co-correct the other two alleles and no prototrophs would be seen. If the C/C mismatch at *his1-672* is recognized and bound, repair at the normal allele (*his1-40* or *his1-30*) would be unidirectional. Moreover, the G/G mismatch at *HIS1-258* might also be expected to co-correct all three alleles and hence hyper-recombination may not be seen. However, these crosses showed HR (Table 14). One explanation for this HR phenomenon could be that the heteroduplex in those diploids (PM300 and PM301) did not cover allele *HIS1-258* and hence there was no mismatch at *HIS1-258*. If the heteroduplex does not cover allele *HIS1-258*, these three-point crosses (PM300 and PM301) may be similar to two-point crosses (PM150: *his1-672 X 1-40* and PM152:*his1-672 X his1-30*)). However, in these crosses (PM300 and PM301) the frequency of parental classes is opposite to that observed in PM150 and

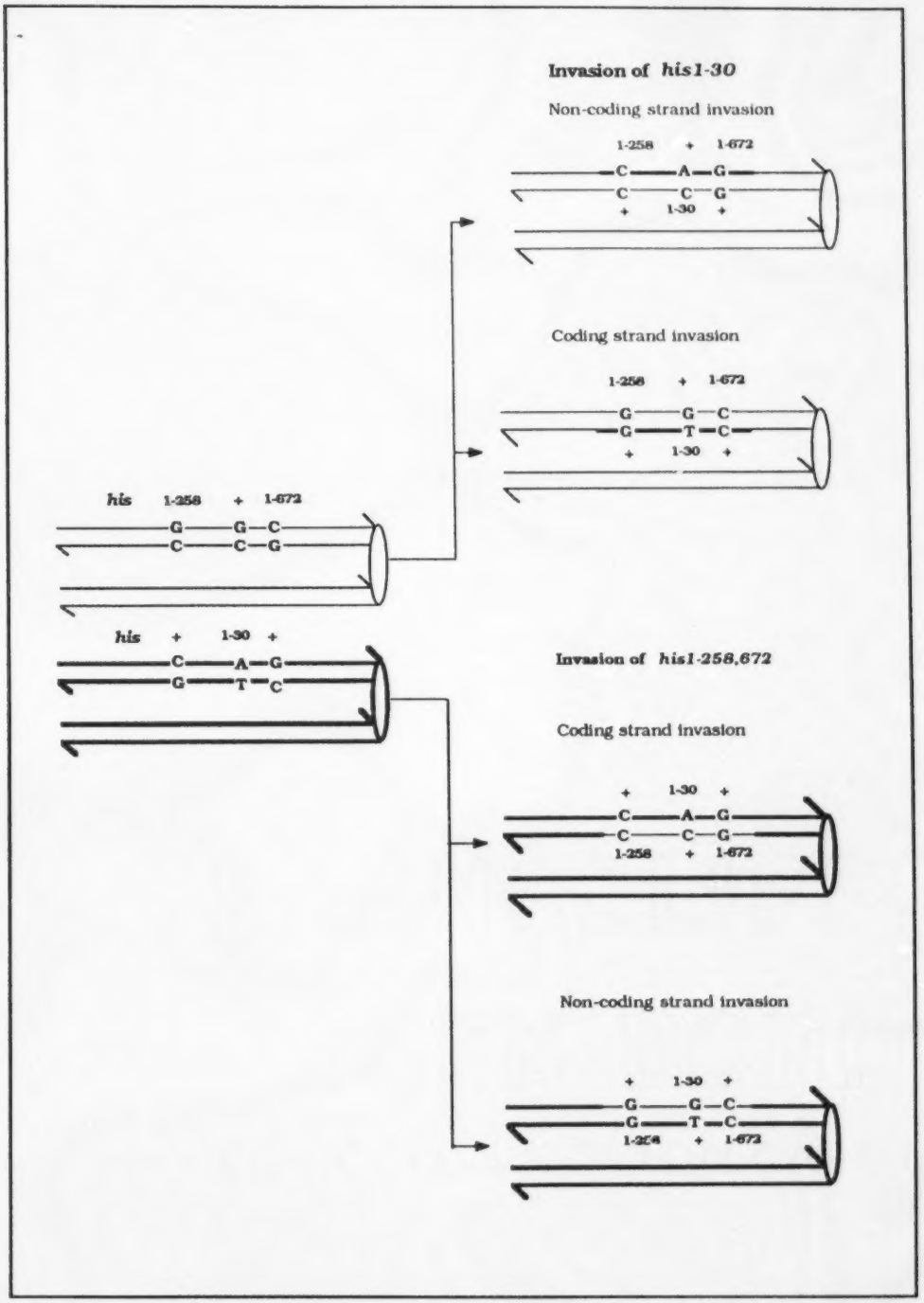

FIG. 31. Heteroduplex formation and types of mismatches in the three-point cross *his1-258, 672* x *his1-30* (PM301) (see the text for details).

PM152 (Table 16). Therefore, exclusion of *HIS1-258* in the heteroduplex may not be the major reason for HR in this diploid.

It seems that the HR phenomenon is unaffected by co-correction from the G/G mismatches at *HIS1-258*. The results of Detloff and Petes (1992) also suggest that co-correction may not affect HR, which is caused by events in a low proportion of tetrads. In a cross between a PMS allele (a palindromic insertion) and a normal allele with a distance of 26 bp, they recorded 85% co-conversions and 6.7% co-PMS. This cross still shows the HR effect. In diploids PM300 and PM301 also, co-correction may not have much influence on the HR phenomenon.

The formation of HR prototrophs in PM300 and PM301 cannot be explained by a single repair event. The following explanations are offered for the results obtained. The greater frequency of P2 type prototrophs over P1 may be explained with two independent repair tracts in the same heteroduplex. In one repair tract the normal allele (*his 1-40*) is repaired unidirectionally as seen in diploid PM150. As the G/G mismatches are repaired well there may be three kinds of repair tracts coming from G/G mismatches at *HIS1-258*. (i) The repair tract may be short and independent of the other repair tract. (ii) The repair tract may be long including alleles *his1-672* and *his1-40* and co-convert both the alleles. (iii) The repair tract may be long and convert *his1-672* after the conversion of *his1-40*. All but the second type can produce prototrophs. As P2>P1 this suggests that some fraction of G/G mismatch repair tracts reach *his1-672* after *his1-40* is corrected. If the second repair tract is on the chromatid which had the *his1-40* allele, the conversion type of event will produce prototrophs and the restoration type of event will produce auxotrophs. On the other chromatid (*his1-672*) this would be the opposite. Depending on the proportion of conversion versus restoration, the frequency of P1 and P2 may vary. If the second repair tract (coming from G/G mismatches) is preferentially the conversion type of repair, the frequency of P2 class prototrophs will be higher than the P1 class. This also depends on the other

variables like preferential formation of heteroduplex on one or the other chromatid or preference in invasion of the coding or the non-coding strand. As the P2 class of prototrophs is greater than the P1 class in diploid PM300, conversion may be greater than restoration. Diploid PM301 showed P1>P2 suggesting that the second repair tract may also be there. The second repair could happen as discussed earlier if the mismatch repair tract frequently ends within 400 bp.

In conclusion, this project was started with the objective of testing the hypothesis that hyper-recombination (HR) could be observed in a cross between a PMS allele and a normal allele if the distance between the alleles is short (4 to 26 bp). The results of the two-point crosses involving a PMS allele and a normal allele show HR if the distance between two allele is short (8 to 20 bp). Three types of PMS alleles (G to C transversion, deletion and palindromic insertion) were tested. All showed HR in the two-point crosses. Selected and unselected tetrad analysis show association of PMS with HR, consistent with the hypothesis. A molecular model was proposed to explain the HR phenomenon. We propose that mismatches at the PMS alleles are bound by proteins and repair does not proceed beyond the recognition step. Unrepaired mismatches segregate postmeiotically. In HR crosses, the proteins bound at mismatches at PMS alleles restrict mismatch repair at normal alleles unidirectionally; repair is otherwise bidirectional. This results in postmeiotic segregation at PMS alleles and repair at normal alleles. The unidirectionality was tested with a variety of three-point crosses involving two PMS alleles and a normal allele. The data from the three-point crosses suggest that one PMS allele protects another allele from co-correction by a normal allele. As the distance between the two PMS alleles is around 400 bp, it seems that occasionally either the heteroduplex or the repair tract does not cover both alleles, so that protection is not seen all the time. An experiment with a short distance (maybe within 50 bp) between two PMS alleles would be preferred since it would be expected to show protection in all the parental

prototrophs (P1 and P2). The results of the last two crosses (PM300 and PM301) are complex and they cannot be explained by the model presented above alone, but they may be explained with two repair tracts in the same heteroduplex.

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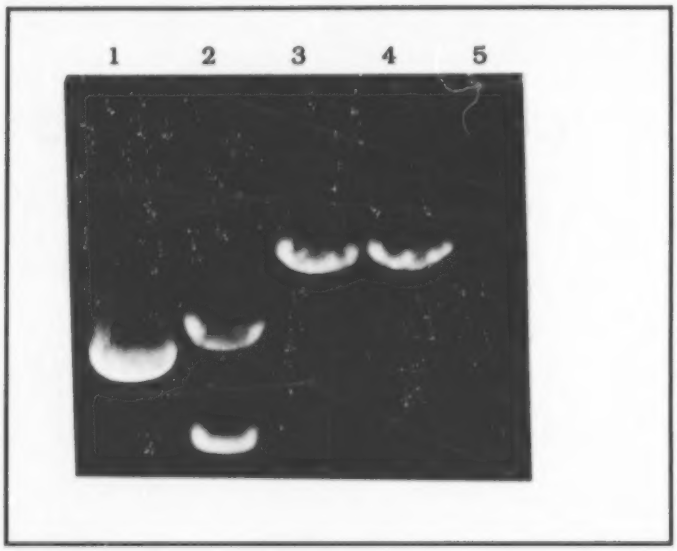
Appendix 1. Agarose gel (0.8%) electrophoresis of restriction digests of the phagemid containing the *HIS1* subclone (pHP1).



The details of the *in vitro* construction of the plasmid pHP1 are given in FIG. 5.

Lane 1. Uncut plasmid (pHP1) DNA.

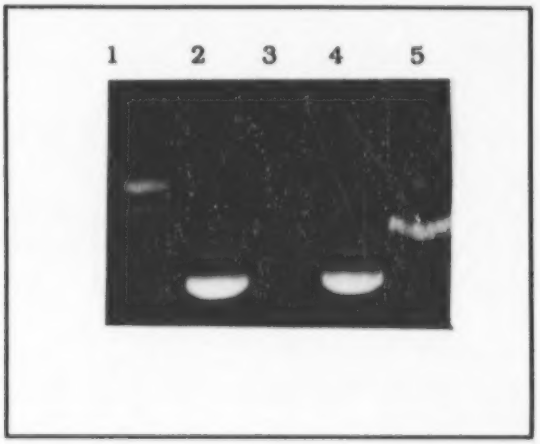
Lane 2. Digestion with *Hin*dIII and *Eco*RI.

Lane 3. Digestion with *Bgl*II.

Lane 4. Digestion with *Eco*RV.

Lane 5. λ DNA /*Hin*dIII marker (the sizes of the fragments, from top to bottom, are as follows: 23.2k, 9.4kb, 6.6 kb, 4.4 kb, 2.3 kb,and 2.0 kb).

Appendix 2. Agarose gel (0.8%) electrophoresis of restriction digests of the plasmid containing the *his1-876* mutation (pHP9).



The details of *in vitro* mutagenesis is described in FIG. 8.

Lane 1. λ DNA /*Hin*dIII marker.

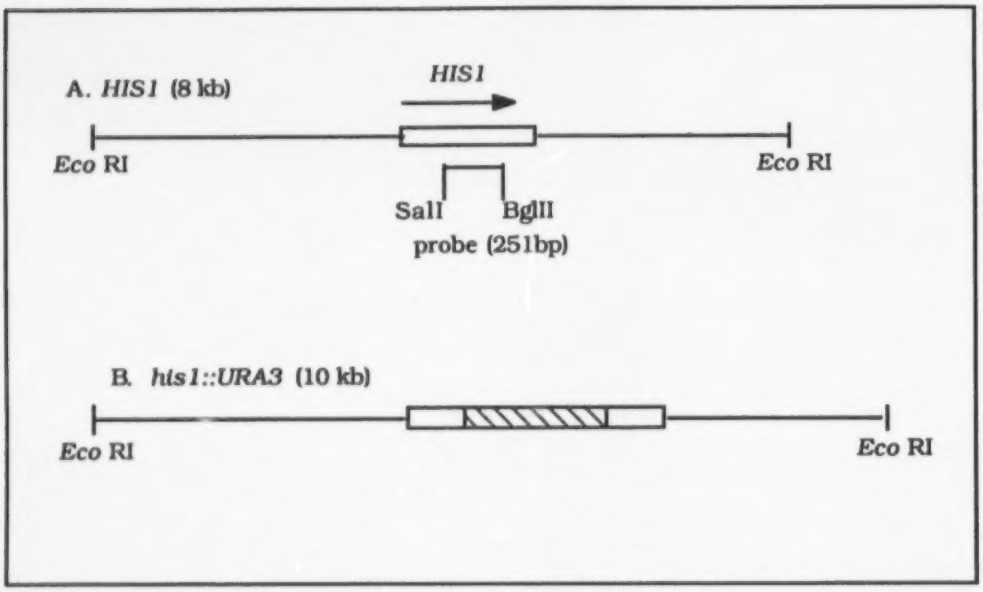
Lane 2. Uncut plasmid (pHP9) DNA.

Lane 3. Restriction (pHP9) with *Apa*I.

Lane 4. Restriction of pHP1 (control plasmid) with *Apa*1.

Lane 5. Restriction of control plasmid with *Eco*RI to show that the control plasmid is restrictable with another enzyme.

Appendix 3. Southern blot analysis of *his1::URA3* disruption.

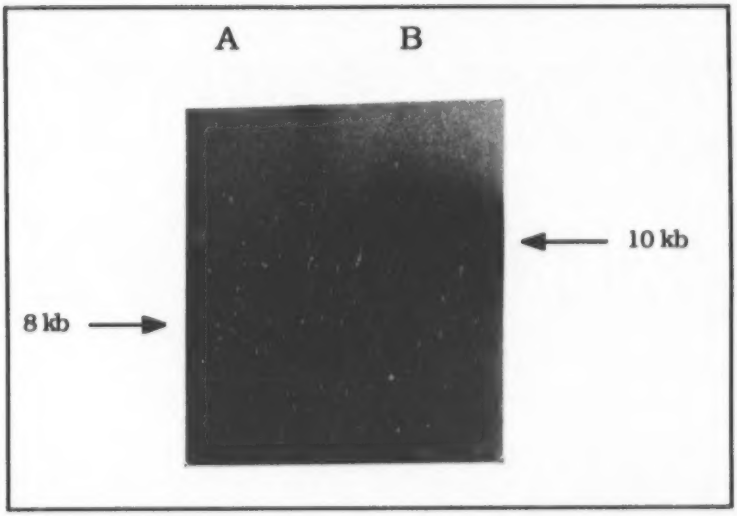


The details of *in vitro* construction of this mutation are described in FIG. 9. Shown on the top (A) is the *HIS1* gene (LZ21-13C). On the bottom (B) is the *his1::URA3* disrpution mutation (PM22). Total genomic DNA was isolated from the yeast strain PM22, by minipreperation procedure of Sherman et al. (1982). DNA was digested with *Eco*RI restriction enzyme. The fragments were resolved on 1% agarose gel. Separated fragements were alkali denatured and transferred to a Gene Screen Plus membrane. The Southern blot was hybridized as recommended by the manufacturer (Du Pont Co.) by using a gel-purified *SalI/BglII* fragment labeled with [^{32}P]dCTP by the random oligonucleotide priming method (Maniatis et al., 1982).

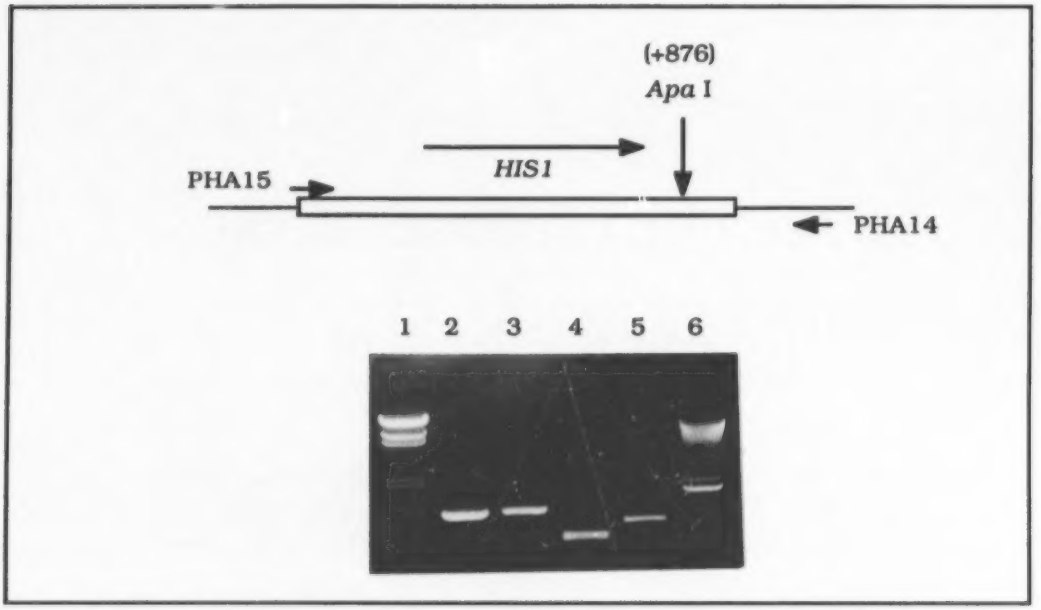
Opposite page: Autoradiograph of Southern blot showing *his1* disruption.

A. Wild type (LZ21-13C).

B. *his1::URA3* (PM22).


A
B
10 kb
8 kb

Appendix 4. Agarose gel (0.8%) electrophoresis of restriction digests of PCR amplified genomic DNA containing the *his1-876* mutation (PM 29).



The details of *in vivo* construction of this mutation (*his1-876)* is described in FIG. 12. Genomic DNA was isolated from PM29 by minipreparation and the fragment containing the *HIS1* gene was amplified by PCR (see the Methods section for details) using two primers PHA15 (-1 to +20) and PHA14 (+1069 to +1080) that hybridize to opposite strands. The amplified DNA (1.08 kb) was run out in 0.8% agarose gel, purified and cut with restriction enzymes.

Lane 1. λ DNA /*Hin*dIII marker.

Lane 2. Uncut DNA .

Lane 3. Digestion of control DNA with *Apa*I.

Lane 4. Digestion of control DNA with *Bgl*II to show that the control DNA can be resticted by some other enzyme.

Lane 5. Digestion with *Apa*I (PM29)

Lane 6. DNA marker (ladder).

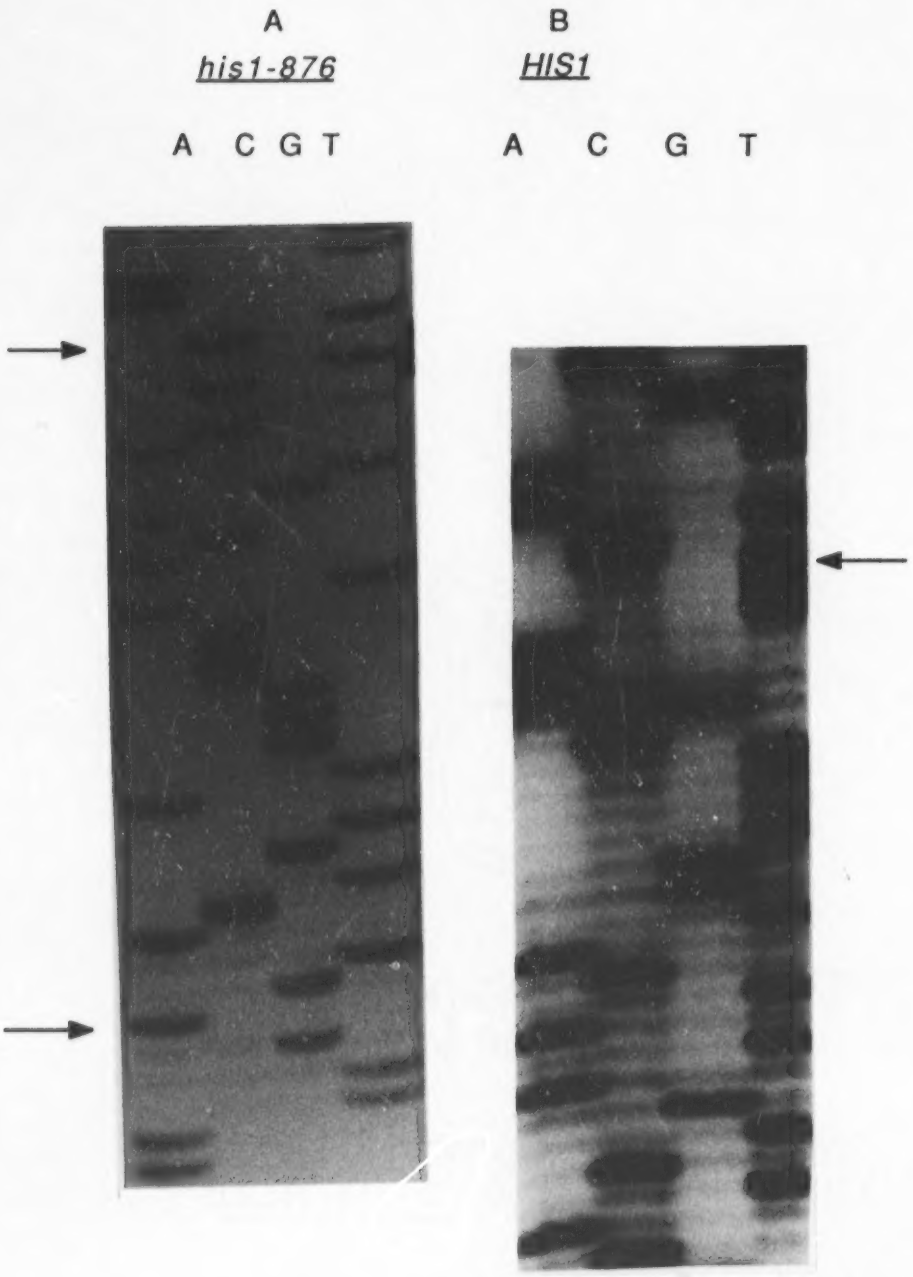
Appendix 5. Autoradiograph of sequencing gels containing the (*his1-876*) mutation.

In order to confirm the base changes introduced on the yeast chromosome, the *his1* fragment of the genomic DNA was amplified by PCR (see appendix 4 for details) and sequenced using a primer spanning +787 to +804 (18 bases) as described in the Methods section.

A. Insertion of 28 bp (between two arrows) creates the *his1-876* mutation.

Inserted sequences are also shown at the bottom.

B. wild -type sequence (LZ21-13C).



WILD TYPE: GAAATTTCTAATT

his1-876: AATT GAGTACTGTATGGG CCCATACAGTACTC TCT